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Volumetric Investigations on Overgrowth (Hypermorphosis) in Chick Embryo Brains

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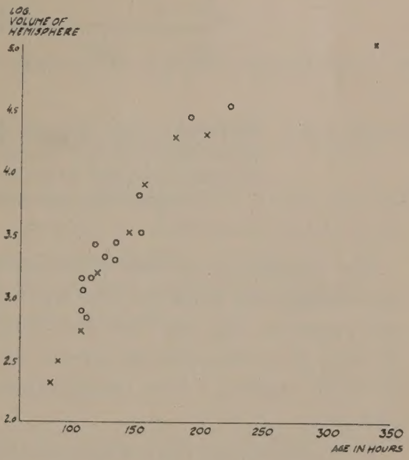
THE present paper is intended as a continuation of preceding publications (Bergquist, 1959 *a*, *b*) on possible causes of the phenomenon of 'overgrowth' (Patten, 1952) in the brain of the chick embryo. Reference should be made to these publications for the relevant literature. In these earlier investigations, it was observed that the mitotic rate in the altered portions of the neural tube seemed higher than in the normal regions, and that the hemispheres and tectum opticum showed marked folding.

The first aim of the present investigation was to compare the volumes of the hemispheres in normal chick embryos and in corresponding stages of embryos with overgrowth, the study being confined to the distinctly evaginated portions of both hemispheres. The embryos measured consisted of those used in the previous work. The surface areas of sections through the walls of both hemispheres were determined planimetrically. In small embryos all sections were measured, and in bigger ones every fifth or tenth. The volume of the wall was then computed from section thickness.



TEXT-FIG. 1. Hemisphere volume (logarithmic scale) in normal chick embryos (*x*) and in embryos showing overgrowth (*o*). The volumes of the embryos with overgrowth lie scattered around the normal ones.

Both normal embryos (designated as *x* in Text-fig. 1) and embryos with overgrowth (designated as *o* in Text-fig. 1) were examined. In Text-fig. 1 the logarithm of the volume of the hemispheres forms the ordinate and the age of the embryos the abscissa. It will be seen that

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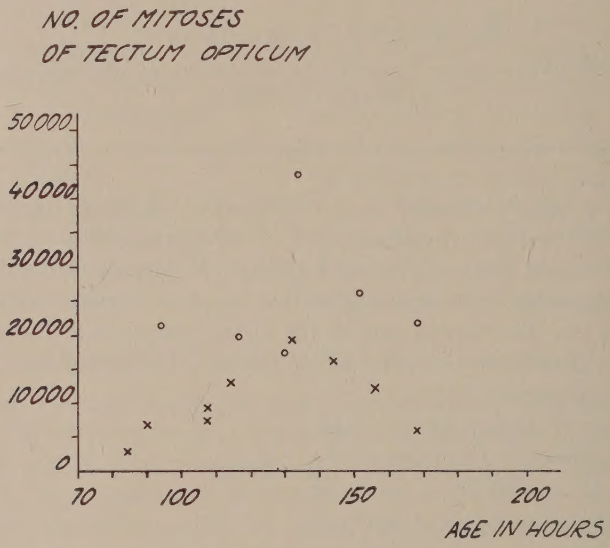
there was no significant difference in hemisphere volume between normal embryos and those with overgrowth throughout the period studied.

The second aim of the present investigation was to study the mitotic number in normal and overgrown chick brains. In order to obtain a well-delimited region the optic tectum was chosen for this study.



Text-fig. 2. Total mitotic number in the tectum opticum in normal chick embryos (*x*) and in embryos showing overgrowth (*o*). The mitotic number of the overgrown hemispheres are obviously higher than the corresponding normal values.

The number of mitoses in the optic tectum was determined in 9 normal and in 6 overgrown embryos and was counted under high magnification in every tenth section. The total number of mitoses in the different embryos is shown in Text-fig. 2 as a function of embryo age in hours. The number is obviously higher in chick embryos with overgrowth than in corresponding stages of normal embryos.

It has previously been demonstrated that an increased number of mitoses next to the ventricular lumen is correlated with evagination of the wall of the neural tube to form bulges during the neuromere phase (Bergquist, 1932; Källén, 1952) or more or less deep furrows during the migration area phase (Bergquist & Källén, 1954). Moreover, by treating the embryos with colchicine, Källén (1953) temporarily arrested the mitotic process so that cells in mitosis aggregated along the inside of the neural tube. This caused the ventricular wall to bulge out in a similar manner to that described here.

The excessive formation of folds in the ventricular wall of embryos with overgrowth might consequently be due, to some extent, to the increased number of mitoses. Another possibility might be that the overproduction of neural tissue within the fixed space available inside the skull had made folding a necessity. But the volumetric studies presented here have shown that overgrowth is not accompanied by a significantly increased volume of the nervous substance. At first sight the higher number of mitoses might seem to be in conflict with this finding. However, it is well known that volumetric growth—particularly in the later stages of development—is mainly due to increase in the volume of individual nerve-cells and to growth of fibres.

SUMMARY

1. There is no increase or decrease of the volume of the hemispheres in brains with 'overgrowth'.

2. The optic tectum of chick embryos showing the overgrowth phenomenon have a markedly higher number of mitoses than do those of normal embryos of corresponding stages.

3. It is suggested that the formation of the overgrowth is fundamentally similar to the development of proliferation furrows and of neuromeric bulges.

RÉSUMÉ

Recherches volumétriques sur l'excès de croissance (hypermorphose) dans le cerveau de l'embryon de poulet

1. Il n'y a pas d'augmentation ni de diminution de volume des hémisphères cérébraux dans 'l'excès de croissance'.

2. Le toit optique des embryons de poulet manifestant le phénomène 'd'excès de croissance' montre une augmentation marquée du nombre des mitoses par rapport aux embryons normaux des stades correspondants.

3. Il est suggéré que 'l'excès de croissance' est dans l'ensemble similaire à la prolifération des sillons et des dilatations neuromériques.

ACKNOWLEDGEMENT

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Respiratory Levels in Mature Sea-urchin Eggs

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INTRODUCTION

For fifty years considerable attention has been devoted to a comparative study of the respiratory metabolism of eggs prior to and after fertilization (Warburg, 1908). With regard to sea-urchin eggs two trends may be recognized. First, variations of oxygen consumption were examined, and although striking increases in respiration were noted in some cases, it was soon recognized that this was not a general phenomenon (Whitaker, 1933). Secondly, studies have been conducted to elucidate the changes in metabolism which follow fertilization, such as the removal of an inhibition in an enzymatic system or the establishment of a new pathway. Runnström has emphasized that a change in the cytochrome chain may take place, an aspect which has been reviewed recently (Runnström, 1956). Krahl (1956) has shown that the balance between glycolysis and the Coenzyme II shunt may vary. Furthermore, experiments with substituted phenols and studies on phosphate uptake or adenosine triphosphatase activity suggest changes in oxidative phosphorylation (Krahl, 1950). The literature has been covered by Rothschild (1956).

The metabolism of the unfertilized egg is generally compared with that of the fertilized or developing egg. However, a comparison of the unfertilized mature egg with its earlier stages of growth in the ovary also appears valid (Borei, 1948). The significance of the low respiration and the apparent inertness of the mature egg has been considered from the latter point of view (Lindahl & Holter, 1943). In Sipunculids too mature eggs and oocytes have been compared with regard to oxidative metabolism (Gonse, 1957).

In the present investigation an attempt has been made to define the factors which influence the rate of exogenous and endogenous respiration in mature eggs of *Strongylocentrotus purpuratus*. Experiments were designed to dissociate levels of respiration in unfertilized and fertilized eggs. The approach taken was to compare intact cells and homogenates in different suspension media. The action of 2:4 dinitrophenol (DNP) under these conditions is also reported.

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MATERIAL AND METHODS

Eggs

The sea-urchin *S. purpuratus* was collected at low tide at the San Juan de Fuca channel. For as long as one month thereafter, eggs were obtained from animals kept in running sea-water at the laboratory. In a few experiments animals stored in the sea at the Friday Harbor laboratories were used.

The animals were induced to spawn by applying a potential of 60 volts to the medium (Harvey, 1956). The eggs were collected in filtered sea-water or sea-urchin coelomic plasma, filtered through cheese-cloth and finally washed three or four times with a hand centrifuge or by sedimentation. Following this the eggs were allowed to stand for 1 to 3 hours in the appropriate medium, after which they were harvested. To prepare a series of aliquots for a Warburg experiment, equal volumes of a thick egg suspension were distributed in graduated centrifuge tubes filled with media. The eggs were packed down and then washed with the solutions to be used in respiration measurements. The final volume was 3 ml.

In order to distribute evenly the thick egg suspension, a syringe was modified by removing the narrow-bore tip. A plastic handle with a set-screw was fitted to this cylinder to prevent the upward movement of the pestle beyond a certain preset value. The plunger was preset to deliver volumes of 0·5, 0·8, or 1 ml. Aliquots for egg counts were also taken in this way. Repeated checking showed that this method was as accurate as and easier than sampling from a dilute suspension. The number of cells delivered by this method did not vary by more than 15 per cent.

Experiments on the respiration of unfertilized eggs were carried out only on batches of eggs which showed a fertilization rate of at least 95 per cent. For measurements on fertilized eggs, sperm were added to a dilute suspension of well-washed eggs until a 95 to 100 per cent. fertilization was obtained; this took about 10 minutes. The sperm were then washed out by centrifugations until spermatozoa could no longer be detected microscopically in the supernatant or at the surface of the eggs. The fertilized eggs were kept at 15° C. for 1½ to 3 hours and then harvested and distributed in aliquots for the Warburg experiment.

Egg counts were made on aliquots fixed in 95 per cent. ethanol and stained with Rose Bengal. The aliquots were diluted 1,700 times in two steps. The last dilution was run on a millipore filter. The cellulose plate was ultimately dried, cleared, and mounted in cedar oil. With the preparation on a transparent grid, the cells were counted over the whole surface of the plate under a binocular microscope (magnification × 45). The number of cells was kept between 300 and 700 per plate. The error on a given plate is insignificant. Larger errors arise in the dilution process, usually of the order of 5 per cent. Variations as great as 15 per cent. were encountered when there were clusters of cells in the aliquot.

Respiration measurements

Respiration was measured at 16° C. with a refrigerated Warburg apparatus. The experiments with unfertilized eggs began 2 to 3 hours after spawning. Vessels with two side-arms were used. The rate of shaking was 116 complete oscillations per minute. The vessels were loaded with 4 ml. of egg suspension, 0·2 ml. of 10 per cent. KOH in the centre well, and 0·2 ml. of solutions in the side-arms. The gas phase was air. The number of cells per flask was usually between 0·5 and 1 × 10^6. In the series of 19 experiments where the respiration of the intact egg in pure sea-water was recorded, the mean number of cells was 0·89 × 10^6, with a standard deviation s = 0·28 × 10^6 (Table 1). In this range of cell concentration a certain damping in the oxygen consumption appears to be unavoidable. An inverse correlation with a coefficient of —0·582, just significant at the 1 per cent. level, exists between cell number per flask and respiration per cell. A similar effect was found in the 5 experiments on fertilized eggs (Table 2). In the case of unfertilized eggs the number of cells cannot be brought much below 0·5 × 10^6 per flask. On the other hand, it is not likely that oxygen diffusion is involved since in our experimental conditions only 10 per cent. of the dissolved oxygen is consumed per hour.

TABLE 1

Seasonal variation in endogenous respiration of unfertilized eggs

Oxygen consumption is expressed per cell per hour. s is the standard deviation.
Medium: sea-water

Month	*Number of cells per flask* × 10^6	*Oxygen consumed* × 10^{-5} μl.	*Month*	*Number of cells per flask* × 10^6	*Oxygen consumed* × 10^{-5} μl.
Jan.	0·85	0·80	Mar.	1·11	0·96
,,	1·50	1·49	Apr.	0·98	0·73
,,	1·21	1·06	,,	0·80	0·68
,,	0·61	1·54	,,	1·30	0·76
,,	0·67	1·18	,,	1·08	0·94
Feb.	0·41	1·49	May	0·59	1·06
,,	0·88	1·11	,,	0·65	1·03
,,	0·72	1·05	,,	1·14	1·16
Mar.	0·62	1·70	,,	0·92	0·82
			July	0·87	0·89
Mean	0·83	1·27		0·94	0·90
s	0·336	0·295		0·224	0·156

As a control a few experiments were done with the diver technique. Stoppered divers with an air volume of 1·2 μl. were used in an accurate thermostat. Fifty to a hundred eggs were placed in each diver so that they lay on the top meniscus of the air-bubble (Gonse, 1954). Values recorded for the oxygen consumption are in agreement with those obtained by the Warburg technique.

With the Warburg technique the figures given for absolute values of oxygen consumption are affected by several errors. An aggregated error of 15 per cent. arises from the uneven partition of cells between the aliquots and from the counting technique. There is also an uncertainty in the estimation of the oxygen consumption, this being done graphically from the pressure change per hour on a Warburg cumulative curve. This last source of error is important for unfertilized eggs in which the rates are small. By and large these errors seem to be much less important as a source of scatter than the fundamental heterogeneity of the material which differs from animal to animal and varies during the reproductive season. To permit comparisons between different experiments the results are expressed as per cent. of a control run in pure sea-water.

TABLE 2

Endogenous respiration of fertilized eggs

Oxygen consumption is expressed per cell per hour. *s* is the standard deviation.
Medium: sea-water

Month	*Number of cells per flask* $\times 10^6$	*Oxygen consumed* $\times 10^{-5}$ μl.
May	0·47	6·25
,,	0·86	4·02
,,	0·58	6·09
July	0·74	5·40
,,	0·36	6·35
Mean	0·60	5·62
s	0·200	0·968

Solutions

The respiration of unfertilized and fertilized eggs has been measured in the following solutions: sea-water at pH 7·5 to 7·8; coelomic plasma at pH 7·4 to 7·7; mixtures of sea-water and sucrose 0·9 to 1·1 M, mixed in equal volumes. Homogenized cells were also studied in sea-water, 0·52 M KCl or 0·9 M sucrose buffered at pH 7·35 with glycylglycine (0·02 M) and supplemented with Na_2HPO_4 (2·5 mM) and eventually with disodium ethylenediaminotetra-acetate (EDTA) (1 mM). As a control intact cells were studied in the same solutions. The sucrose medium was contaminated with about 5 to 10 per cent. of sea-water, this being due to the difficulty of washing the cells in a pure sucrose solution. The 'isotonic' sucrose medium was theoretically iso-osmotic with the local sea-water, but was actually somewhat hypertonic for the sea-urchin eggs. In this sucrose solution a shrinkage of the cells was observed both in the absence or presence of glycylglycine or EDTA. The cell diameter decreased from 83 ± 6 μ to 73 ± 6 μ in the unfertilized eggs, and from 81 ± 3 μ to 72 ± 4 μ in the fertilized eggs.

Coelomic fluid was withdrawn from the perioral region of female sea-urchins with a hypodermic needle. Depending on the size of the animal and the repletion of its digestive tract 30–80 ml. of fluid can be obtained by this method. The fluid was centrifuged at 20,000 g. for 15 minutes and the supernatant (plasma) decanted. Plasma was prepared daily from the female which had also supplied the eggs. This procedure was necessary since, following freezing and drying, difficulties were encountered in dissolving the preparation.

The following compounds were examined for their action on respiration: glucose (0·02 M), pyruvate (0·02 M), succinate (0·02 M), and 2:4 dinitrophenol (DNP) buffered at pH 7 (0·02 mM). Phosphate was estimated in coelomic plasma or sea-water by the method of Berenblum & Chain (1938).

Homogenization

Complete homogenization of unfertilized eggs was accomplished by forcing the suspension 10 times through a 22-gauge needle or in a special homogenizer described by Dounce (1955). For fertilized eggs only the latter procedure has been employed since these cells are more difficult to disrupt. The homogenization was carried out approximately 1 hour following the start of the Warburg experiment. The cells were removed from the flask, homogenized within 3 minutes at 16° C., and then returned to the flask. Four minutes were needed to bring the system to temperature equilibrium. Thus the first reading could be taken 5 minutes after homogenization.

Viability

Fertilization and development were normal for 80 to 100 per cent. of the cells following experiments in sea-water. On the other hand, cells washed and suspended in sea-water after exposure to plasma, sucrose, or KCl media, showed a somewhat smaller recovery; development proceeded to the blastula stage in 50 to 80 per cent. of the cells, and further development was infrequent.

RESULTS

Normal respiration

Measurements of endogenous respiration of unfertilized eggs were made during the reproductive season from January to July in sea-water. The nineteen values recorded can be observed to fall into two groups (Table 1). The first group, found between January and March, had a mean endogenous respiration rate 40 per cent. higher than that of the second group ($P < 0·01$). Since a damping of the oxygen consumption is directly related to increases in cell number, calculations were made using the mean number of cells per flask for these two groups. As seen from the values in Table 1 the difference between these means is not significant. Thus eggs with different endogenous oxidative capacities may be distinguished. It may be mentioned here that a lowering of the endogenous respiration was reported in *Phascolosoma* oocytes upon resumption of growth in the spring (Gonse, 1956, 1957). Measurements with the diver technique, early in January, gave the following figures for the unfertilized eggs' respiration in sea-water: 1·26, 1·49, and $2{\cdot}32 \times 10^{-5}$ μl. O_2/hour/cell. These values are in agreement with the data of Table 1.

Endogenous respiration of fertilized eggs was measured in sea-water for 3 hours' duration; the experiments were started 2 hours after fertilization (Table 2). During this period the eggs were passing from stage 1 ('lake' stage, with a large clear area in the centre of the egg) to the 8- or 16-cell stage. However, respiration was stable enough and began rising only towards the end of the experiment. The data show that the mean value of oxygen consumption was 6·2 times greater than that of unfertilized eggs.

In an attempt to approximate conditions to those in the ovary the respiration of unfertilized eggs was examined in coelomic plasma. Although, in the animal, eggs are associated with somatic cells and ovarian fluid, it was nevertheless felt that these experiments would be of interest since they would give some indication of the exogenous oxidative capacity.

There is rather little information available on the coelomic fluid of sea-urchin. Its salt content appears to be similar to sea-water (Harvey, 1956). Inorganic phosphate was found in a concentration of 0·06 to 0·36 mM; the higher value being obtained from the fluid of animals starved in the laboratory. By comparison 0·01 mM phosphate was present in the natural sea-water used in these experiments. Data on the content of polysaccharides, free sugars, proteins, and amino-acids have been reported (Lasker & Giese, 1954; Messina, 1957). It was found here that a small amount of proteins can be precipitated by 10 per cent. trichloracetic acid or by dialysis against distilled water at refrigerator temperature. Also, a pigment was present in the plasma and had a spectrum-like echinochrome (Harvey, 1956). The plasma took on a brown colour on standing in the laboratory, but appeared red immediately on removal from the animal or when acidified. The pH of the brown plasma was 7·6 and a spectrum showed maxima

TABLE 3

Exogenous respiration of unfertilized eggs

Respiration of control material in pure sea-water is taken as 100. From January to February, 8 experiments; from February to July, 6. Range of values given in brackets

	In plasma, no washing	*In plasma, sea-water washing*	*In sea-water with glucose* (0·02 M)
January to February	192 (150–332)	124 (104–150)	136 (129–146)
February to July	103 (103–104)	110 (105–120)	126 (103–146)

at 275 and 395 mμ and 3 others between 450 and 600 mμ. The plasma alone simulates a small gas uptake of 500 μl./hour/ml. when prepared according to the above procedure. It was necessary to subtract this value from the results.

A positive effect of the coelomic plasma on respiration was observed regularly on eggs laid from January to February, that is on cells belonging to the group with a higher endogenous respiration (Tables 3 & 4). Plasma had no effect on eggs obtained later in the season. Another phenomenon which can be observed from Table 3, and particularly from Table 4, is the inhibitory effect on respiration in plasma caused by washing for 2–3 hours with sea-water. Eggs laid and washed in plasma during the same period always have a greater oxygen consumption. Temporary starvation, removal of some factors by washing, or the modification of the cell membrane apparently impairs normal exogenous respiration. The addition of an ovarian extract, however, restores respiration to its normal level. The extract was obtained from a homogenate of ovary in sea-water and centrifugation at 100,000 g. for 20 minutes in the Spinco ultracentrifuge. The supernatant utilized in the experiment did not consume oxygen. A partial inhibition of exogenous respiration by washing with sea-water has also been observed in the oocytes of *Phascolosoma* (Gonse, 1957).

TABLE 4

Respiration of unfertilized eggs in plasma from fresh and starved animals

Respiration of control material in pure sea-water is taken as 100. Experiment in January. Eggs from the same fresh animal

	In plasma from fresh animal	*In plasma from starved animal*	*In sea-water with glucose* (0·02 M)
Eggs not washed by sea-water	233	119	. .
Eggs washed 3 hours by sea-water	149	110	144
Same eggs after addition of ovarian homogenate	220	172	165

Glucose added in sea-water consistently stimulated respiration of the unfertilized eggs, both in winter and spring eggs. When respiration in pure sea-water was taken as 100, the mean respiration in the presence of glucose was calculated to be 130 (Table 3). Succinate or pyruvate were without effect when added either in sea-water or plasma. The experiment reported in Table 4 would indicate that glucose by itself does not explain the augmented respiration and that other factors which are absent in the fluid of starved animals may be required.

These findings suggest that another source of variability in the intensity of exogenous respiration in plasma may be accidental or seasonal fluctuations in the level of substrates or cofactors in coelomic fluid. This may contribute to the lack of stimulation of respiration by plasma in late eggs.

On the other hand, from Table 1 it would appear that there exist two kinds of eggs defined by differences in the intensity of their endogenous respiration, a fact which may be related to their different responses to factors present in plasma. Although these two kinds of eggs are equally fertile and do not show morphological differences, they were grown under different seasonal conditions. The first group could be considered as immature from the viewpoint of metabolism. At the stage studied (haploid after the second division of maturation) the early eggs retain the active metabolism that characterizes growing oocytes.

Action of sucrose and 2:4 dinitrophenol (DNP)

The great inertness of the mature unfertilized eggs, particularly late eggs, is evident in our experiments. This well-known phenomenon could be attributed largely to two causes: first, to a high degree of impermeability peculiar to the haploid stage, and, second, to the impairment or uncoupling of metabolic reactions. These two possible mechanisms may be interrelated, the lower activity of metabolism being a limiting step for the uptake of exogenous factors. Experiments have been performed bearing on these two aspects. To act on the cell membrane, thereby possibly modifying permeability, eggs were treated with solutions low in salts such as slightly hypertonic or iso-osmotic sucrose. With respect to the second problem, studies on the effect of DNP on respiration were undertaken.

TABLE 5

Effect of hypertonic sucrose on unfertilized eggs' respiration

Sucrose (1 or 1·1 M) added to an equal volume of sea-water or plasma. Respiration of control material in pure sea-water is taken as 100. From March to May. Range of values given in brackets

Sea-water–sucrose	*Sea-water–sucrose with glucose* (0·02 M)	*Sea-water with glucose* (0·02 M)	*Plasma*	*Plasma–sucrose*
103 (67–135)	146 (118–230)	131 (107–146)	113 (106–120)	213 (176–248)

Effect of sucrose media on egg respiration. The results obtained with a mixture of sea-water and 1 or 1·1 M sucrose were variable (Table 5); the endogenous respiration was activated in some cases and inhibited in others. On the other hand, a mixture of plasma-sucrose (1:1 vol.) greatly stimulated respiration in spite of the fact that the eggs used in these experiments belonged to the second group and consequently did not show exogenous respiration in pure plasma. Also sucrose somewhat enhanced the positive effect of glucose on respiration.

The medium used in these experiments was hypertonic and produced morphological changes in the cells. The variable results obtained on endogenous respiration may be a reflection of irregularities in the morphological alteration. In general a finely wrinkled cell surface and the appearance of clear spaces in the periphery due to plasmolysis were observed. Such changes are partially reversible; after washing with sea-water, over 50 per cent. of the eggs can still be fertilized and will develop, although yielding thick-walled blastulae. These results point to a rather inconsistent effect of hypertonicity on the cell membrane or cortex. The permeability would be expected to increase in some cases, thus permitting penetration of substrates which could then stimulate respiration. However, the fact that endogenous respiration is always altered, when individual experiments are considered, indicates some action on metabolism rather than a simple modification of the cell membrane. Sucrose itself, in substrate concentration, was without effect on respiration.

The susceptibility of endogenous respiration to sucrose treatment was studied further. Experiments were carried out with iso-osmotic sucrose in the absence of sea-water in order to examine the effect of salt and water removal from the external medium (Table 6). This medium was buffered and supplemented with various amounts of disodium ethylenediaminotetra-acetate (EDTA) to chelate the bivalent cations of the small amount of sea-water introduced into the sucrose together with the eggs. In sea-water alone the presence of EDTA does not modify respiration. However, in sucrose high concentrations of EDTA depress the oxygen consumption of fertilized eggs (Table 6).

TABLE 6

Effect of iso-osmotic sucrose on the respiration of fertilized and unfertilized eggs

Sucrose 0·9 M, phosphate 2·5 mM, glycylglycine 0·02 M, pH 7·35. Respiration of control material in pure sea-water is taken as 100. From May to July. Range of values given in brackets. EDTA = disodium ethylenediaminotetra-acetate

	EDTA concentration		
	0–0·1 mM	1 mM	5 mM
Unfertilized eggs	159 (132–196)	177 (149–266)	183 (169–206)
Fertilized eggs	99 (99)	91 (87–99)	56 (55–58)

In this sucrose medium the unfertilized eggs show a consistent activation of endogenous respiration, whereas in the fertilized eggs an inhibition of respiration was produced if sufficient EDTA was present. The morphological effect of iso-osmotic sucrose is less conspicuous than that of the hypertonic sucrose–sea-water solution. In both unfertilized and fertilized eggs plasmolysis was not noticeable, and the finely wrinkled aspect of the cell surface was less evident. But a shrinkage of 12 per cent. in diameter occurred. Furthermore, in fertilized eggs there was an increase of the perivitelline volume and disappearance of the hyaline layer in 30 minutes. Segmentation was completely blocked in sucrose, but it was resumed when the eggs were returned to sea-water. It is significant to note that sucrose has identical morphological effects on the cell surface but gives opposite results with respect to respiration of fertilized and unfertilized eggs.

In summary: the oxygen uptake tends to reach a level that is intermediate between those which normally characterize these two types of cells. A possible interpretation is that two different respiratory systems are involved. One is present in both fertilized and unfertilized eggs, and this is activated by sucrose treatment. The other, active only in fertilized eggs and responsible for their higher normal respiration, is inhibited in sucrose solution, perhaps because of the absence of calcium ions. As can be seen when comparing Tables 5 and 6, the activating effect of iso-osmotic sucrose on unfertilized eggs was not due to its slight hypertonicity for the cells but more likely to removal of salts. Further, an isotonic solution of KCl (0·52 M), buffered and supplemented with EDTA, depresses the respiration of both fertilized and unfertilized eggs.

Effect of dinitrophenol on respiration. The addition of DNP (0·2 mM) was made 1 to 2 hours after the beginning of the experiments and subsequent readings were taken during the following hour. Full activation of respiration generally occurred within 10 minutes and was stable thereafter except in the presence of glucose or in the sucrose medium where the effect tended to reach a maximum and then to slacken. For unfertilized eggs in pure sea-water the increase of respiration due to DNP action was about 3 times greater than the endogenous respiration (Table 7). This was valid if DNP was added either in sea-water or in sucrose. Thus the activating effect of sucrose is additive with that of DNP. However, if glucose was present in the sucrose medium, the respiratory increase was not as great. Pyruvate, succinate, and glutamate had no such counteracting effect, nor had glucose when the experiments were carried out in sea-water.

TABLE 7

Effect of 2:4 dinitrophenol (DNP) on respiration of unfertilized eggs

The sucrose medium is as in Table 6. The values represent the ratio: $(R_{X+DNP}—R_X)/R_{SW}$, where R_X is the respiration in the various media, R_{X+DNP} is the respiration after addition of DNP, and R_{SW} is the respiration of control eggs from the same batch in sea-water. (E), EDTA 5 mM; (G) glucose 0·02 M; (PSGl) pyruvate succinate and glutamate 0·02 M in the medium. Range of values given in brackets. DNP 0·2 mM

Medium:	*Sea-water*	*Sucrose*	*Plasma*	*Sea-water with*	*Sucrose with*
Ratio:	3·1 (2·6–3·7) 2·6 (E)	3·1 (2·5–4·1) 2·9 (E)	6·7 (6·1–7·4)	3·1 (G) (2·4–3·9)	1·4 (G) (1·2–1·6) 3·1 (PSGl)

The enhancement of the DNP effect in coelomic plasma appears remarkable, since the magnitude of the activation was about 7 times the value of the endogenous respiration. Thus, when plasma is supplemented with DNP, the unfertilized egg respiration becomes slightly greater than that of the fertilized egg.

Other treatments have also been able to show a high oxidative capacity of unfertilized eggs (Runnström, 1949). It thus appears that there is a deficiency of endogenous intermediates and/or cofactors in unfertilized eggs which can be supplied by coelomic plasma. The nature of these compounds which permit full development of DNP activation is unknown. From Table 7 it can be seen that succinate, pyruvate, glutamate, and glucose do not act in this capacity. It may be recalled that with the eggs used in these series of experiments (late eggs) plasma has virtually no stimulatory effect on respiration in the absence of DNP. But impermeability is apparently not concerned in this lack of response. DNP has no action on gas uptake by plasma in the absence of eggs.

TABLE 8

Effect of DNP on respiration of fertilized eggs

The sucrose medium is as in Table 6. Values are expressed as the ratio in Table 7. (E) EDTA 5 mM; (G) glucose 0·02 M; (S) succinate 0·02 M in the medium. Range of values given in brackets. DNP 0·2 mM

Medium:	*Sea-water*	*Sucrose*	*Sea-water with*
Ratio:	1·6 (0·9–2·3) 0·9 (E)	1·7 (1·2–2·3) 0·4 (E) (0·1–0·7)	1·2 (G) 1·6 (S)

For comparison some data are given in Table 8 for fertilized eggs. Here endogenous respiration was increased by 1 to 2 times its value by DNP. Bearing in mind that upon fertilization the respiration increases about sixfold, these figures would then represent an increase of ten- to twentyfold over endogenous respiration of unfertilized eggs. It may be further mentioned that EDTA (1 to 5 mM) inhibited DNP activation. This was specially clear for fertilized eggs suspended in sucrose only. Thus it appears that cations are necessary for full DNP action or penetration.

Activation of respiration by DNP appears to be largely dependent on the availability of certain oxidizable substrates. These are stored in limited amounts in mature unfertilized eggs which have a low endogenous respiration, but can be supplied by an external medium such as plasma and oxidized if an uncoupling agent is present. Thus the endogenous respiration of unfertilized eggs is inhibited at the same time by a deficiency in substrates and by a deficiency in acceptors for oxidative phosphorylation. It may be that as growth comes to a stop adenosine di-phosphate (ADP) is no longer provided by synthesis requiring adenosine tri-phosphate (ATP). Fertilization triggers off an accelerated formation of oxidizable substrates. Metabolism assumes a complete endogenous character, thus permitting an increase of respiration.

Respiration of whole homogenate

It has been suggested that fertilization could cause a rearrangement or the closer association of particles in the eggs. The presence of a physical barrier within the cell might explain the inertness of the unfertilized egg and thereby its inactivated metabolism. Data have been reported on the respiration of homogenates of *Arbacia* eggs (Keltch *et al.*, 1950) which are in favour of this suggestion. In the presence of succinate, oxygen consumption by this preparation was, on a cell to cell basis, as great as that of the intact fertilized eggs. Also, the possible role of calcium ions in controlling an acceleration of oxygen uptake has been investigated by Hultin (1950) on homogenates of *Paracentrotus* eggs.

In the present work the respiration of whole homogenates and not that of special fractions was investigated. Respiration was measured within 5 minutes after the completion of the homogenization procedure. Different media were tested with the criterion of preservation of the morphology of cell particles and maintenance of respiration. But in whole homogenates other critical aspects have also to be taken into account. For instance, homogenization here entails a tenfold dilution of biological material; this decreases the concentration of substrates and separates enzymatic sites. Another significant problem was the possibility that toxic and inhibiting substances are released. In general it is true that after such an homogenization the medium is rather poorly defined.

The homogenization media tested were: natural sea-water, sucrose, and KCl. These were buffered at pH 7·35 by glycylglycine and supplemented with phosphate and various amounts of EDTA.

Regarding morphological characteristics as examined under the microscope (oil immersion), the best media were found to be sucrose and KCl supplemented with EDTA 5 mM. The need for a high concentration of EDTA may be due to the release of ions, particularly calcium, after homogenization. The form and size of the particles such as mitochondria and yolk drops did not appear significantly different from that found in the intact cell. There was no or only little aggregation of these particles and no cytoplasmic coagulum. Furthermore, such preparations could be stored overnight in the cold without change. In sea-water or without EDTA, yolk drops expand and burst, and there was clustering of particles and coagulation of the cytoplasm.

Despite identical appearance of the KCl and sucrose homogenates, their rates of respiration were quite different. KCl gave poor and inconsistent results. The respiration was always lower at all phases than in sucrose and sometimes completely inhibited. The sucrose and KCl media differed only by their content of potassium and chloride, and these ions may therefore be concerned in the inhibition observed. In subsequent work the KCl medium was discarded and homogenization was carried out in sucrose and also, for comparison, in sea-water. The presence of various amounts of EDTA (0 to 5 mM) did not affect the respiration. Table 9 shows that oxygen consumption, after the first 20 minutes, was about the same in sucrose or sea-water and was thus unaffected by the salt content. In fact, in the whole sucrose homogenate the amount of inorganic salts released from the cell may not be negligible. For instance, assuming that half of the potassium of eggs is released in the medium, this would yield a final concentration of 5 mM.

TABLE 9

Respiration of homogenates of unfertilized and fertilized eggs

Means obtained from 9 experiments. Respiration of intact eggs of same animal, in pure sea-water, is taken as 100. Values for fertilized eggs are multiplied by 6. Sea-water or sucrose (0·09 M) are buffered at pH 7·35 by glycylglycine (0·02 M), supplemented with phosphate 2·5 mM, EDTA 1 to 5 mM. (S) succinate 0·02 M and (G) glucose 0·02 M added. Range of values given in brackets

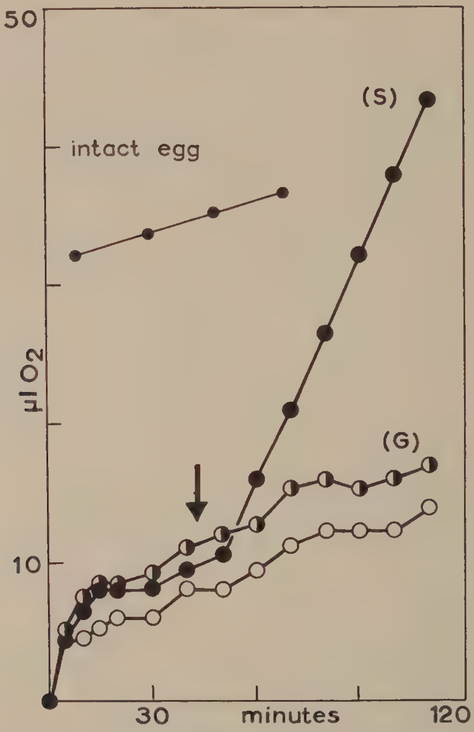
	First phase		*Second phase*	
	In sea-water	*In sucrose*	*In sea-water*	*In sucrose*
Unfertilized eggs	278 (153–339)	481 (225–883)	145 (77–249)	100 (86–131)
Fertilized eggs	288 (216–402)	462 (288–720)	162 (90–240)	140 (90–204)

The problem of dilution by homogenization is an important one if one is to compare the rates of respiration in intact cells and in homogenates. To estimate this effect various concentration of cells from 0·36 to 1·27 × 10^6 per flask were used. No correlation was found between the number of cells homogenized and the intensity of respiration.

The rate of endogenous respiration of homogenates was not constant with time. The change in rate was generally not progressive but actually abrupt, indicating two different activities (Text-fig. 1). Thus one can distinguish an initial respiration (phase 1) and, later, a second lower respiration (phase 2).

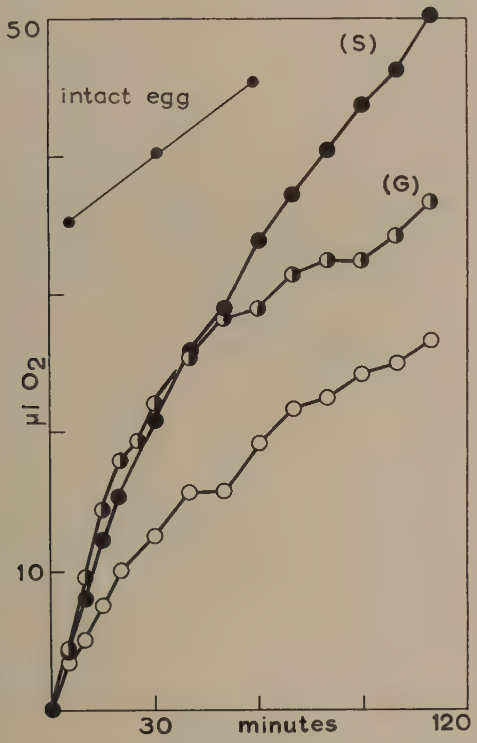
The duration of phase 1 was variable as it ranged from 10 to 50 minutes, but generally it was of about 20 minutes' duration. It was not possible to correlate its duration with experimental factors such as EDTA concentration or cell number. Its possible correlation with the length of the period of starvation of the cells before the beginning of the experiment was not studied. This instability may be related to the inactivation of some enzymes or cofactors or may correspond to the disappearance of a specific substrate stored in limited and various amounts. The respiratory rate per hour, for phase 1, was calculated from data obtained in the first 20 minutes. This method evidently tends to give values lower than the true initial rate of oxygen consumption in phase 1, since the rate progressively decreases and the duration of the phase is sometimes less than 20 minutes. After phase 1 the endogenous respiration of the homogenate proceeded at a constant rate for 1 or 2 hours (Text-fig. 1). Later, this respiration also slackened progressively unless substrate was added.

In Table 9 the means calculated from a series of 9 experiments are given. To permit a comparison between fertilized and unfertilized eggs the sixfold increase of respiration at fertilization has been taken into account, and thus the oxygen consumption is expressed in terms of the endogenous respiration of intact unfertilized eggs. Regarding the intensity of respiration, homogenates obtained from unfertilized or fertilized eggs gave surprisingly similar results. Furthermore, their behaviour in sea-water or in sucrose also differed in the same way, i.e. the initial endogenous respiration (phase 1) is higher or is maintained for a longer time in sucrose than in sea-water, but little difference is found for phase 2 respiration.



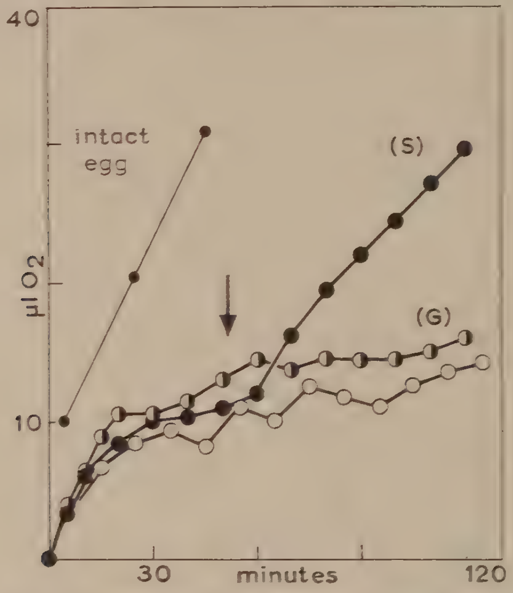
Text-fig. 1. Unfertilized eggs. Respiration of whole homogenate in iso-osmotic sucrose. Arrow indicates addition of glucose 0·02 M (G), succinate 0·02 M (S) or medium (open circles). Respiration of same number of intact eggs is in sea-water.

The similarity is emphasized by a study of the effect of succinate (0·02 M) and glucose (0·02 M). A sample of these experiments is given in Table 10 and it can be seen that no significant difference is observed in homogenates obtained either from fertilized or unfertilized eggs. Respiration could be stimulated by succinate to a constant high rate (Text-figs. 1, 2, 3). This effect could be obtained either in phase 1 or 2 (Text-figs. 1, 2). Apparently succinoxidase was well preserved in these homogenates as in the preparation of Ycas (1954) in phospate 1/15 M at ph 7·1. The addition of glucose during phase 2 was without effect. Also, in an experiment on unfertilized eggs the presence of ATP and hexokinase did not modify this result. On the contrary, glucose alone stimulated the respiration during phase 1 (Text-fig. 2). Pyruvate (0·02 M) was without effect at all phases, even in the presence of catalytic amounts of succinate. DNP at a concentration of 0·2 mM did not affect the rate of respiration in phase 2.



Text-fig. 2. Unfertilized eggs. Respiration of whole homogenate in iso-osmotic sucrose. Glucose 0·02 M (G), succinate 0·02 M (S) are already present in the medium at the time of homogenization. Respiration of same number of intact eggs is in sea-water.

The presence of the transient high endogenous respiration immediately after homogenization was of interest because the rate of this respiration is of the same order as that of the intact fertilized egg. It may therefore merit further study. Our observations suggest the participation of glycolysis, since glucose stimulates this system. However, glucose does not maintain it, and, furthermore, pyruvate alone does not increase respiration as would be expected. Although succinate was rapidly oxidized here it does not give a respiratory level as high as that of the intact fertilized egg. Under the experimental conditions employed, it appears



Text-fig. 3. Fertilized eggs. Respiration of whole homogenate in iso-osmotic sucrose. Arrow indicates addition of glucose 0·02 M (G), succinate 0·02 M (S), or medium (open circles). Respiration of same number of intact eggs is in sea-water.

Table 10

Effect of addition on the respiration of homogenates from fertilized and unfertilized eggs

Respiration of same intact eggs, in pure sea-water, is taken as 100.
Media and additions as in Table 9

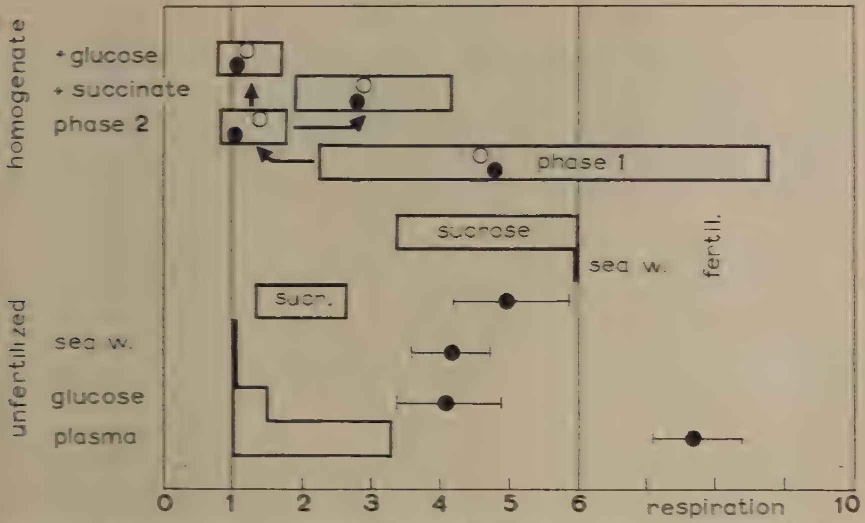
	In sucrose	*In sucrose with glucose*	*In sucrose with succinate*
Unfertilized eggs:			
phase 1	225	346	403
phase 2	86	88	420
Fertilized eggs:			
phase 1	69	92	147
phase 2	15	12	46

that the high endogenous respiration of the fertilized egg is dependent on the integrity of cellular structure. In the homogenate the stable endogenous level is the low one which is characteristic of the intact unfertilized egg.

CONCLUSIONS

The data on respiratory rates in different experimental conditions are summarized in Text-fig. 4.

Starting from the lower level, the base line is the oxygen consumption of the intact mature unfertilized egg suspended in sea-water. This endogenous respiration is about $1 \cdot 10^{-5}$ µl. O_2/hour/cell and is taken as unity. Apart from the small effect of glucose, the exogenous respiration is negligible during the greater part of the reproductive season. The stable endogenous respiration of a whole homogenate of fertilized or unfertilized eggs prepared in sucrose or in sea-water is also found at this level.



TEXT-FIG. 4. Summary of respiratory levels. Intact fertilized egg in sea-water is at level 1, intact fertilized at 6. Black circles are the respiration of intact unfertilized eggs with addition of DNP. For the homogenates, black circles are the means of the results obtained with unfertilized eggs and the open circles the means with fertilized eggs. Length of rectangles corresponds to scattering of data.

Higher intensities of respiration, termed levels 2 or 3, can be obtained in the presence of glucose or exogenous factors of the plasma in hypertonic solutions. These levels are normally exhibited by early eggs which have a distinct exogenous respiration in plasma. Levels 2 or 3 tend to be attained in endogenous conditions by a treatment with iso-osmotic sucrose and EDTA, in both unfertilized and fertilized eggs. The respiration of homogenates of fertilized or unfertilized eggs prepared in sucrose also tends to settle at this level in the presence of succinate.

A higher state of respiration, termed level 4, is obtained by addition of DNP to intact unfertilized eggs in sea-water.

Employing the same terminology, the level of respiration of fertilized eggs is 6, that is, 6 times greater than the endogenous respiration of unfertilized eggs. The initial respiration of homogenates of fertilized or unfertilized eggs in sucrose, and also the exogenous respiration of the unfertilized eggs in plasma when activated by DNP, are also found at level 6.

The highest level of respiration was the DNP activated endogenous respiration of fertilized eggs. This is at a level of about 15.

These values should be considered essentially as guide-marks. The establishment of identical respiratory levels in different experimental conditions does not, of course, imply an identity in metabolic pattern.

Some of the results can also be considered with respect to the comparative metabolism of unfertilized and fertilized eggs. In previous studies on *Phascolosoma* oocytes the importance of the interrelation between endogenous and exogenous respiration has been stressed (Gonse, 1957). Egg maturation appeared as the end-point of a progressive modification in the balance between these two oxidative capacities. In this respect fertilization can be considered to be a process which breaks this relationship, and which permits the establishment of a new type of equilibrium and, eventually, of a rise in endogenous respiration. Some evidence is given here in favour of such a phenomenon in sea-urchin eggs: the unfertilized eggs are able to oxidize exogenous substrates from the plasma, either directly (early eggs) or in the presence of DNP (late eggs), but they are deficient in endogenous oxidizable substrates.

However, the situation of the mature sea-urchin egg is rather different from that of mature *Phascolosoma* eggs. In the sea-urchin the maturation goes as far as the completion of the second division, so that the cells studied here are haploid. Previous investigations showed that in these cells the endogenous respiration is lower than in the diploid stage (Lindahl & Holter, 1943). A study on this haploid stage thus does not actually bear on the metabolism of the oocyte at the end of its growth; rather it describes a more inert and transient stage which, in other forms, is of shorter duration and not amenable to experiments.

It may be that no major change in the enzymatic equipment is involved at the time of fertilization, but essentially the modification of one or few reaction equilibria, some of them having been further shifted during the maturation process. The possible role of ions released in the cell or entering it following a change in permeability at the time of fertilization should not be underestimated. The experiments on homogenates do not show any spectacular differences between unfertilized and fertilized eggs. The two types of preparations showed no difference in oxygen consumption and response to substrates. The higher respiration of the intact fertilized egg seems to be bound to the structure of the cell and is probably inhibited by the removal of divalent cations such as calcium. It is tempting to locate this system in the cell cortex, which is directly susceptible to the effect of sucrose and where a birefringence change normally follows fertilization and is antagonized by hypertonic solutions (Monroy, 1957). A rise in phosphate uptake occurs also at fertilization (Whiteley, 1949) and there is evidence that a phosphorylating mechanism is present at the cell surface (Lindberg & Ernster, 1948; Lindberg, 1950; J. B. Litchfield & A. H. Whiteley, personal communication). The unstable system which operates immediately after homogenization and which has a high oxygen consumption and utilizes glucose may be related to these metabolic activities of the cell cortex.

SUMMARY

1. A study of the respiratory capacity of unfertilized and fertilized eggs of *Strongylocentrotus purpuratus* was undertaken in a variety of suspending media such as sea-water, coelomic plasma, sucrose, and KCl. The action of glucose, succinate, and dinitrophenol on the respiration of intact eggs and homogenates of eggs was reported. In this manner it was possible to define a number of relative levels of respiration.

2. By suspending intact eggs in sucrose or by homogenization it was possible to decrease the difference in respiratory levels between fertilized and unfertilized eggs.

3. By employing a rapid homogenization technique two levels of respiration could be distinguished: an initial rapid rate which progressively decreases over a 20-minute interval, followed by the establishment of a constant rate of respiration at a lower level.

4. These results are discussed with regard to the problem of the metabolic changes which take place upon fertilization.

RÉSUMÉ

La consommation d'oxygène des œufs fertilisés ou non fertilisés de *Strongylocentrotus purpuratus* a été mesurée dans différents milieux tels qu'eau de mer, plasma coelomique, solutions de sucrose ou de KCl. On a étudié l'effet sur la respiration d'œufs intacts ou homogénéisés, du glucose, du succinate et du dinitrophenol. Un certain nombre de niveaux dans l'intensité de la respiration ont été ainsi définis.

La différence de consommation d'oxygène entre œufs fertilisés et non fertilisés peut être diminuée par homogénéisation ou par traitement des cellules intactes avec une solution de sucrose.

Après une homogénéisation rapide on peut distinguer deux niveaux respiratoires: une consommation d'oxygène initiale élevée qui décroît pendant les premières vingt minutes, puis une respiration plus faible et stable.

Ces résultats sont revus brièvement en fonction du problème d'un changement métabolique suivant la fertilisation.

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Osteolathyrogenic Effects on the Developing Rat Foetus[1]

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WITH ONE PLATE

SKELETAL and aortic changes in foetal rats treated with osteolathyrogenic agents via the maternal system throughout pregnancy or very late in pregnancy have been described by Stamler (1955). Developmental changes in *Xenopus* larvae (Chih, Witschi, & Ponseti, 1954) and in salamander and toad larvae (Levy, 1955) have also been described. The abnormalities produced in these forms by these agents have apparently been related to a direct effect of the agent on the mucopolysaccharide ground substance. No attempt has been made in a mammal to determine if there are specific time-susceptible periods in embryogenesis when this type of stimulus may induce true teratogenic effects. The present study was devised to determine the effect of a known osteolathyrogenic agent during specific stages of gestation and especially to observe the effect of this agent during the early critical stages of embryogenesis.

MATERIALS AND METHODS

Cystamine dihydrochloride was used as the lathyrogenic agent. It has proven activity (Dasler & Milliser, 1958), is obtainable commercially in a pure form (from Nutritional Biochemicals Corp., Cleveland, Ohio), and is a stable compound. The animals were fed by gavage to regulate the amount and time of feeding. Rats fed 100 mgm. of cystamine daily fared very poorly and usually succumbed within 3 or 4 days, but when the dosage was reduced to 50 mgm. of cystamine daily over a 5-day period the animals remained in good health. Five groups of pregnant rats of the Sherman strain were fed 50 mgm. of cystamine in 1 ml. of saline by gavage for 5 consecutive days of gestation beginning on days 7, 9, 11, 13, and 15. Control animals were fed 1 ml. of normal saline by gavage beginning on day 14 for 5 consecutive days. All animals were allowed the regular Purina rat chow and water *ad lib*. The rats were killed on the day of term and the

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foetuses fixed in 95 per cent. alcohol, eviscerated, cleared in 2 per cent. KOH, and stained with Alizarin red. Two specimens from each litter were fixed in Bouin's fluid and kept for further examination. Resorption sites were sought and recorded.

RESULTS

The feeding of cystamine increased foetal resorption beyond that found in control rats as shown in Table 1. No gross abnormalities in the fresh or bone-stained foetuses of the control group were found. The only structural abnormalities noted in the experimental series were defects in rib structure and a rather marked kyphosis in some animals (Plate, figs. 1, 2, 3). The rib defects were first manifest in the series of rats treated on days 9–13 as a rather subtle twisting of the medullary region of the rib (Plate, fig. 3). In the series of rats treated later in gestation this basic rib defect increased in severity so that even more pronounced deformities appeared (Plate, fig. 2). The defect appeared in all members of an affected litter in about the same degree, even though there was some variance in the extent of the malformation among litters treated on the same days of gestation. The more posterior ribs were affected most and the lesions were almost always bilaterally symmetrical in each foetus. Although the curvatures of the ribs tended to be uniform in distance from the mid-saggital plane within a given litter, there was enough variation of this distance among the various litters to indicate that the site of deformity was not dependent upon the site of a single muscle attachment.

TABLE 1

Days of treatment	*Number of mothers*	*Living foetuses*	*Resorption sites*	*Percentage resorption*
Controls 14–18	5	51	2	3·9
7–11	5	40	9	18·6
9–13	7	47	13	21·6
11–15	5	42	9	17·6
13–17	5	39	15	27·7
15–19	5	37	13	26·0

The most severe deformities appeared in those animals treated on days 15–19 of gestation. These foetuses showed a marked kyphosis, foreshortening of the thoracic cage, and severely altered rib morphology involving all of the ribs bilaterally (Plate, fig. 2). It is of special interest to note that none of the animals treated on days 7–11 of pregnancy produced foetuses with skeletal or other gross abnormalities.

Microscopic examination of the affected ribs revealed rather marked disorganization of the osseo-cartilaginous model as compared with controls (Plate, figs. 4, 5, 6). The core appears to be pushed toward the periphery of the developing rib especially near the point of greatest distortion. In addition, there is a considerable proliferation of the connective tissue immediately surrounding the model. This again is most evident in the areas of greatest distortion.

DISCUSSION

It is apparent that this osteolathyrogenic agent should not be considered to have a true teratogenic effect on the rat foetus. In spite of the unusual rib patterns mentioned above, there has been no basic developmental alteration in rib morphology or in the skeleton as one might expect to find with a true teratogen. The critical stages of teratogenic susceptibility in the rat are generally considered to be during the 8th to 13th days of gestation. Nevertheless, the gross deformities apparent at birth and after skeletal staining in those animals treated in the later stages of gestation necessitate some classification. The stimulus used here is effective at some time between the period of true teratogenic susceptibility and the immediate prenatal period. For lack of any present terminology with which to label these types of agents it is suggested that the term ‘foetal pathogen’ be used.

Cartilaginous models of all ribs in the rat are present on day 15, and ossification begins in ribs 3–9 on day 16 of development and has begun in all ribs by day 17½ of development (Wright *et al.*, 1958). This would explain the failure to produce any effects during the 7–11-day period, and the slight changes in those rats treated from day 9 to 13. Metabolic interference with the mucopolysaccharide ground substance during these stages of ossification could account for much of the torsion appearing in the osseo-cartilaginous models at term. Further histochemical studies are needed to analyse this point.

SUMMARY

1. Foetal rats exposed to an osteolathyrogenic agent, cystamine, in the later stages of gestation revealed marked deformities of the thoracic ribs.

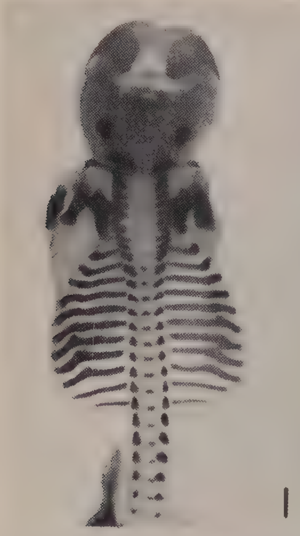
2. Foetuses exposed during the so-called critical stages of embryogenesis in early gestation showed no abnormalities at birth.

3. It is suggested that the term ‘foetal pathogen’ be used to describe those stimuli which affect foetal growth patterns after the period of teratogenic susceptibility and which produce marked variations from the normal at birth, to distinguish them from the ‘foetal teratogen’ which produces basic alterations in organogenesis.

RÉSUMÉ

Actions ostéolathyrogéniques sur le fœtus de rat en voie de développement

1. Des fœtus de rat exposés à l’action d’un agent ostéolathyrogénique, la cystamine, aux stades avancés de la gestation, montrent des déformations nettes des côtes thoraciques.



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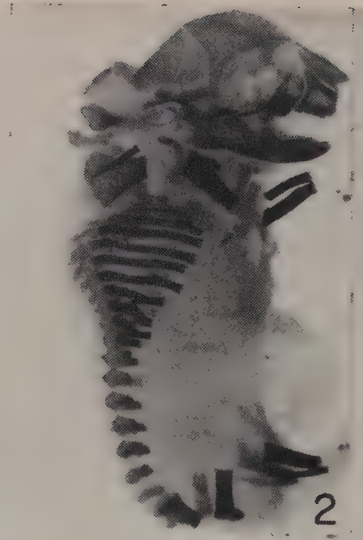

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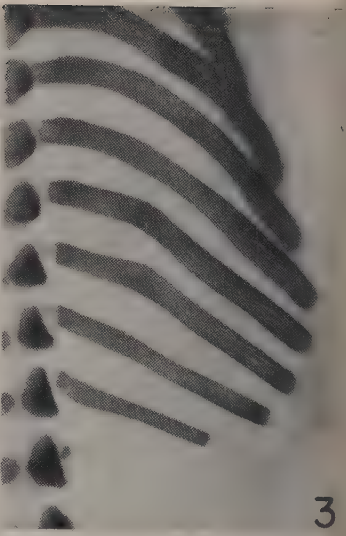

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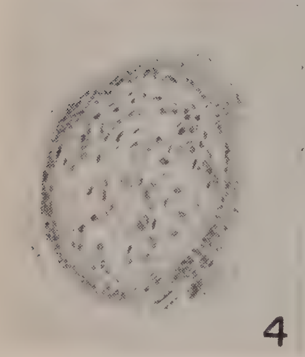

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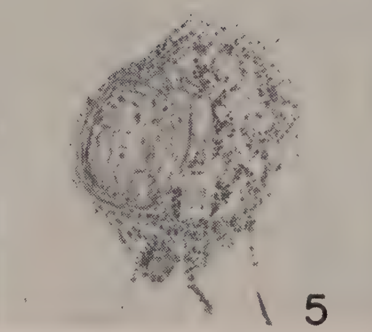

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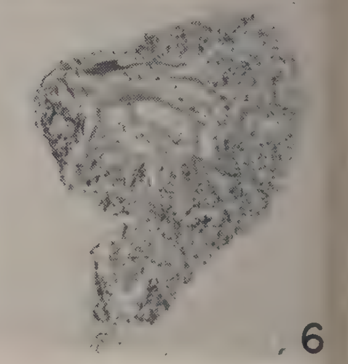

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2. Des fœtus exposés à la même action pendant les stades prétendus critiques de l'embryogenèse, c'est-à-dire au début de la gestation, ne montrent aucune malformation à la naissance.

3. Il est suggéré que le terme 'action fœtale-pathogène' soit utilisé pour qualifier les stimuli qui affectent les rythmes de croissance fœtaux après la période de sensibilité tératogène et qui produisent des modifications marquées après la naissance, par opposition aux actions 'fœtales-tératogènes' qui provoquent des altérations fondamentales de l'organogenèse.

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EXPLANATION OF PLATE

Fig. 1. 18-day foetus showing distortion of ribs. Treated from days 11–15. × 4.

Fig. 2. Term foetus showing kyphosis and rib distortion. Treated from days 15–19. × 3.

Fig. 3. Term foetus showing minimal rib distortion resulting from treatment on days 9–13. × 8.

Fig. 4. Cross-section 10th thoracic rib from control foetus. Gomori's trichrome stain. × 80.

Fig. 5. Cross-section 10th thoracic rib of foetus treated on days 15–19. Gomori's trichrome stain. × 80.

Fig. 6. Cross-section of same rib as shown in fig. 5, through area of maximum distortion. Gomori's trichrome stain. × 80.

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Acetylcholinesterase Activity in the Regenerating Forelimb of the Adult Newt, *Triturus*

by MARCUS SINGER, MARJORIE H. DAVIS, *and* ELEANOR S. ARKOWITZ[1]

From the Department of Zoology, Cornell University

WITH ONE PLATE

INTRODUCTION

THIS paper continues the analysis of the role of acetylcholine in the important influence which the nerve exerts on regeneration of a body part in amphibians (reviewed by Singer, 1952, 1959*a*). Without the nerve, regeneration does not occur. In previous studies it was shown first of all that classical blocking agents of acetylcholine activity—atropine sulphate, procaine hydrochloride, and tetraethylammonium hydroxide—suppress regeneration and delay its resumption for a significant time (Singer, Davis, & Scheuing, 1960).

In a second work which assayed the content of acetylcholine in the developing regenerate, the substance was found in abundance within the growth at all stages of its development (Singer, 1959*c*). Indeed, during the early formative phases of growth, when development is most dependent upon the nerve, the amount of acetylcholine within the regenerate rose substantially above that of the non-regenerating limb and declined only later during the time of histogenesis. The cycle in content of acetylcholine and the blocking effect of classical acetylcholine inhibitors suggested that acetylcholine may be the agent of the nerve in its control of the early events of regeneration, a conclusion already advanced for planarian regeneration by Welsh (1946). However, there are alternative explanations of our results which were summarized in preliminary fashion in a review by one of us (Singer, 1959*b*; see also above references). But, whatever the role acetylcholine may play in regeneration of a body part, the fact of the high content raises the additional and important question of the means whereby such a level is attained and maintained. There are a number of possible explanations for the variation in acetylcholine during development, the most salient of which, variation in the content or activity of the well-known hydrolytic enzyme acetylcholinesterase, is herewith analysed by biochemical and histochemical means.

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The results show, indeed, that the abundance of acetylcholine within the growth can be correlated inversely with the activity of the esterase. Moreover, the results demonstrate an interesting chemical aspect of regeneration hitherto unsuspected and contribute to our understanding of biochemical mechanisms during regenerative development.

MATERIAL AND METHODS

Regenerates of a given stage (stages summarized by Singer, 1952) were removed and homogenized. They were tested for the esterase by one of two methods which employ acetylcholine chloride as the substrate. In one case, the extent of splitting by the esterase was measured gasometrically with a manometer (evolution of carbon dioxide with increase in the acetic acid of the solution), and, in the other, colorimetrically after reacting the residual acetylcholine with hydroxylamine to form hydroxamic acid. The second method was more sensitive and the first served only to confirm the results. Although the first procedure will be described, only brief mention will be made of it in the results.

Bilateral amputations were performed routinely, as in past studies from this laboratory, through the distal third of the upper arm. The operative and postoperative care of animals has also been reported repeatedly. Approximately 10 days after amputation, at a time when regenerative enlargement is first grossly visible at the amputation surface, and thereafter regenerates were selected for assay according to the stage of their development. The growths were removed under a dissecting microscope, care being taken to exclude the proximal adult tissues. Regenerates of the same stage from a number of animals, and often from both sides of the same animal, were homogenized together to provide a measurable amount of enzyme activity. In the younger stages as many as 38 regenerates taken from 19 or more animals were used for determinations.

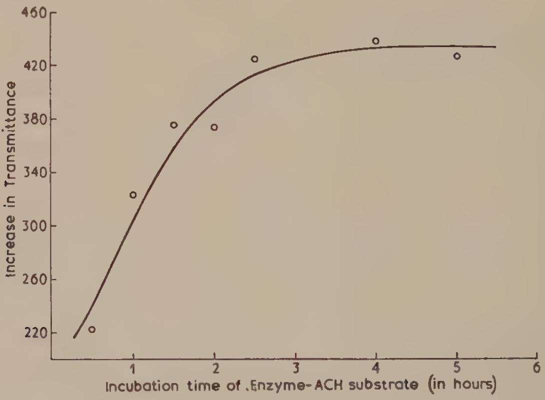
The method of colorimetric determination

The Hestrin (1949) method yielded a reproducible series of assays of young and old regenerates. The procedure depends on the interaction of acetylcholine with hydroxylamine (H_2NOH) as follows:

$$RCOOR' + H_2NOH \longrightarrow RCONHOH + R'OH,$$

where R and R′ represent respectively the acetic acid and choline of acetylcholine. Hydroxylamine reacts with acetylcholine stoichiometrically in alkaline pH to yield acethydroxamic acid. Ferric chloride is then added and forms a coloured (purple brown) complex with the acid. After development of the colour the optical density of the solution is read at 540 $m\mu$. The details of the procedure are described adequately in Hestrin's (1949) original work; but it is necessary to recount some of the procedure here in order to record variations and other items of interest in our determinations of the cholinesterase content of regenerates.

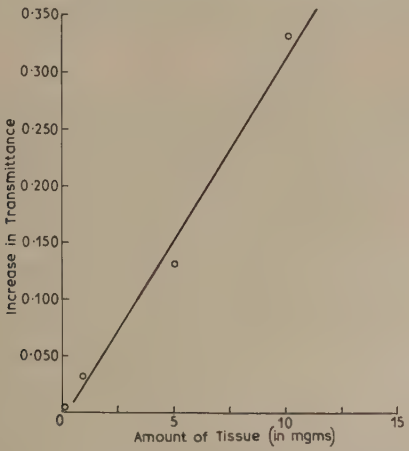
We used 1·75 N sodium hydroxide instead of the recommended 3·5. The pH of the resulting solution was approximately 7·2 instead of 11, and at this pH a minimum of approximately 1 hour was required for the reaction of acetylcholine and hydroxylamine (37·5° C). However, only half the recommended amount of hydrochloric acid sufficed to bring the solution to the pH necessary for the formation of the iron complex. We used 0·5 ml. acetylcholine of a 0·008 M solution and added 0·5 ml. of homogenate solution to attain the final recommended volume for incubation.



TEXT-FIG. 1. The relation between incubation time of enzyme-substrate and enzyme activity. Activity is expressed as increase in transmittance of an acetylcholine solution resulting from hydrolysis of acetylcholine (see text).

The tissue immediately upon weighing was homogenized in a known volume of cold buffer, usually 1·0 ml. or less. The buffer solution consisted of $NaH_2PO_4.6H_2O$, 0·05 M; $MgCl_2.6H_2O$, 0·02 M; NaCl, 0·1 M; and it was adjusted to a pH of 7 with NaOH solution. This pH is within the optimum for enzymatic hydrolysis of acetylcholine (Hestrin, 1950). The homogenate was centrifuged and the supernatant stored for a brief period at 4° C.

For assay an amount of solution containing 10 mg. of tissue was removed (generally in about 0·1 to 0·2 ml.) and diluted to a volume of 0·5 ml. with 2·8 per cent. fresh gelatin solution, the gelatin presumably increasing the stability of the enzyme (Hestrin, 1950). Then 0·5 ml. of acetylcholine solution was added and the mixture incubated for 2 hours (37·5° C); 2·0 ml. of the alkaline hydroxylamine were then added. The combined solution was incubated at 37·5° C. for 2 additional hours. The time of 2 hours' incubation and 2 hours' reaction with hydroxylamine was selected after a number of tests were run at various times. Then concentrated hydrochloric acid (0·5 ml.) was added, followed by ferric chloride, and the volume brought to 5 ml. and read spectrophotometrically. Text-fig. 1 shows the relation between incubation time and enzyme activity expressed as change in light absorption (multiplied by 100) due to declining amount of acetylcholine. The increase in transmission due to hydrolysis of the acetylcholine solution by the homogenate was determined as the difference between the transmission of homogenate and control solutions. Control solutions were treated exactly like the experimental one, adjusted to the same volume, but without added homogenate. The incubation time did not affect the acetylcholine content of the control. In Text-fig. 2 the relation between substrate concentration and enzyme activity is depicted.



Text-fig. 2. The relation between concentration of homogenate and enzyme activity. Activity is expressed as increase in transmittance of an acetylcholine solution resulting from hydrolysis of acetylcholine by a serially diluted homogenate of regenerate tissue (see text).

In addition to the above control, a blank reading was always first made of a solution containing all substances except the enzyme and acetylcholine solutions, which were replaced by water. This reading served as 100 per cent. transmission or 0 per cent. absorption. A turbidity reading was also made of a mixture in which the enzyme solution was inactivated by adding it after hydrochloric acid rather than before. The reduction in transmission due to turbidity was 0·076; it was considered a constant for all runs and it is therefore not included in the calculations.

The homogenates proved enzymatically so active that only a fraction was used for assay; consequently, from 4 to 8 runs were made in each sample. In most instances the readings conformed with one another; but in a few the deviation was great.

The Warburg manometric method

This method is reviewed by Mendel & Hawkins (1950) and only some features are described here. The regenerates were weighed rapidly and then homogenized in 0·9 ml. of cold buffer (NaCl, 0·15 M; $MgCl_2$, 0·04 M; $NaHCO_3$, 0·025 M). The homogenate was transferred to a Warburg vessel (5 ml.); and 0·1 ml. of fresh acetylcholine solution (0·4 M) was introduced into the side arm. The vessel was cooled in a water-bath at 23° C., gassed with nitrogen containing 5 per cent. CO_2, and equilibrated for 20 minutes. The solutions were mixed, the vessel shaken (132 times/min.), and readings taken periodically. Controls for non-enzymatic hydrolysis and for homogenate respiration were run simultaneously.

Histochemical studies of cholinesterase

Attempts were made to localize acetylcholinesterase within tissues of the regenerate at various stages of development. Studies were made on 22 regenerates distributed as follows among the stages: 8 early regenerates, 3 medium, 4 late to palette, and 7 advanced palette or early digital stages (see stages of Singer, 1952). The cholinesterase was visualized according to the method of Koelle (1950) as modified by Chessick (1954). The limb-stump and regenerate were removed without anaesthesia. The bone was then teased from the stump and the tissue fixed overnight (for about 8 hours) in cold buffered formalin (1 part 40 per cent. formaldehyde to 9 parts M/15 phosphate buffer of pH 6·5–6·8). The specimen was washed for 10–30 minutes and impregnated in 10 per cent. gelatin (4 hours at 37° C.). It was fixed again in formalin for 10–30 minutes. Frozen sections were made at 10–25 μ. Staining, mounting, and dehydration procedures are described by Koelle (1950). Sections were generally counter-stained with 0·5 per cent. methylene blue. The site of activity was visualized by the precipitation of copper thiocholine; subsequent treatment with ammonium sulphide converted the crystals to copper sulphide (Malmgren & Sylvén, 1955). A positive reaction was a golden tan to dark-brown colour. In the histochemical tests the incubation media and procedures suggested by Koelle (1950) were used to identify all cholinesterases or, selectively, specific acetylcholinesterase and non-specific cholinesterase. According to these procedures, non-specific cholinesterase is inhibited by low concentrations (10^{-6} M) of di-isopropylfluorophosphate (DFP), whereas specific acetylcholinesterase requires much higher concentrations.

The tests were as follows:

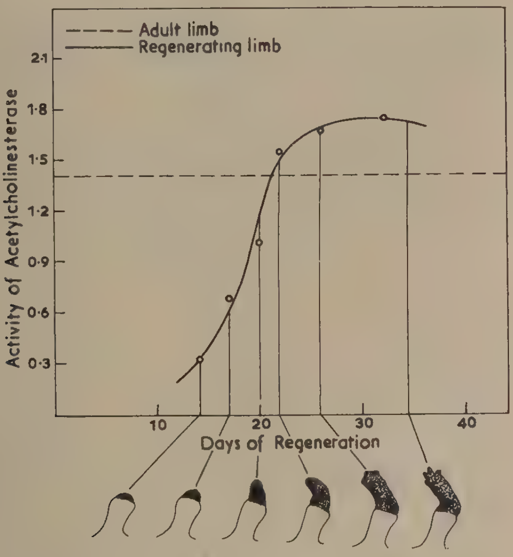
Incubation solution	*Enzyme identified*
A. Acetylthiocholine	All cholinesterases
B. Acetylthiocholine + DFP (10^{-6} M)	Specific acetylcholinesterase
C. Butyrylthiocholine	Non-specific cholinesterase
D. Butyrylthiocholine + DFP (10^{-6} M)	DFP resistant non-specific esterases

Control slides were incubated in DFP (10^{-3} M) for 30 minutes and then tested as for 'all cholinesterases' (*A* above). The DFP in this concentration destroys all cholinesterases, including specific acetylcholinesterase.

RESULTS

The acetylcholinesterase of normal and regenerating forelimbs

Table 1 lists the results of enzymological tests of the activity of cholinesterase in normal and regenerating limbs of various ages. In Text-fig. 3 the average activity is plotted against the age in days and approximate stage of development. The average activity is expressed per unit of dry weight of regenerate. The reason for expressing the results in this way has already been given in a preceding work on the acetylcholine content of the regenerate (Singer, 1959*c*), but should be touched upon here. In the present assay for cholinesterase activity the determinations were always made on 10 mg. of tissue. However, the young regenerate is quite edematous and the water content is substantially higher than the older



TEXT-FIG. 3. The activity of the enzyme is expressed as loss in light absorption of the ferric hydroxamic acid complex due to hydrolysis of the acetylcholine substrate. All values are based on dry weight ×10 (see Table 1, last column). Sketches are included of representative regenerative stages, from left to right: early bud, medium bud, late bud, palette, early digital, and medium digital. The age of each stage is an average one based on many previous experiences. Non-stippled area is the amputation stump; the border is the original amputation line. Sketches about ×3 actual size.

one. Consequently, the enzyme activity was diluted by the abundant fluid. If the activity is expressed in dry weight the influence of water content on the activity can be controlled and the various stages can now be compared directly with one another.

TABLE 1

The activity of cholinesterase

Stages of regeneration	*No. homogenates*	*No. animals per homogenate*	*Activity 10 mg. wet tissue*	*Average activity*	*Average activity in 10 mg. dry tissue × 10*
Early bud	2	7 17	0·041 0·045	0·043	0·33
Medium bud	2	8 19	0·097 0·079	0·088	0·69
Late bud	2	19 18	0·151 0·149	0·150	1·01
Palette	2	17 11	0·217 0·220	0·219	1·55
Early digital	2	8 11	0·302 0·296	0·299	1·67
Medium digital	2	9 9	0·308 0·306	0·307	1·71
Normal limb	3	2 2 2	0·333 0·334 0·330	0·332	1·42

The activity of the enzyme is expressed as loss in light absorption of the ferric hydroxamic acid complex due to hydrolysis of the acetylcholine substrate (see text for calculations of dry weights).

The dry weight of the regenerate at each stage of development was reported in a previous work (Singer, 1959*c*). Since there was a direct relation between optical transmission and cholinesterase activity (Text-fig. 2), the results could be recalculated to read transmission per 10 mg. dry weight instead of 10 mg. wet weight (see Table 1). The final value was multiplied by 10 in order to obtain a convenient value for plotting (Text-fig. 3). Therefore, the plot on Text-fig. 3 represents the activity of the enzyme per 100 mg. of regenerate. The corrections for water content were based on the following percentages of dry weight per individual stage taken from Singer (1959*c*): normal forelimb without bone, 23·4 per cent.; early regenerate bud, 12·8 per cent.; medium bud, 12·8 per cent.; late bud, 14·8 per cent.; palette, 14·1 per cent.; and early digital regenerate, 17·8 per cent. An example of the calculations is as follows for the early regenerate bud: 0·043 (the measured increase in transmission due to enzyme hydrolysis by the 10-mg. sample of wet tissue) multiplied by the fraction 100/12·8 yields the expected increase in light transmission due to enzyme activity in a sample representing 100 mg. dried tissue.

The turbidity of the homogenate decreased the extent of light transmission. Consequently, each determination should be higher, roughly according to the content of dried tissue; in the case of the early regenerate bud the additional amount should be about half of that for the normal limb, the turbidity reading of which was 0·076.

The results depicted in Table 1 and Text-fig. 3 show that the activity of acetylcholinesterase is very low during the early formative phases of regeneration, but then climbs rapidly during the stages of the late bud and during early differentiation. In the early regenerate bud and perhaps in a still earlier stage, there is a small but measurable amount of cholinesterase. Wound tissues in the early post-amputation days were not measured. Beyond the stage of the early bud the enzyme activity per unit of tissue increases slowly at first and then more rapidly as differentiation of muscle and other tissue sets in. During the latter time the enzyme activity tends to rise above that of normal; still later, it presumably falls and eventually levels off near the normal value.

We were unable to measure the enzyme activity in the early phases of regeneration with the Warburg method. However, in the later stages to which the technique was adequately sensitive, there was a rise in activity which paralleled that already described with the Hestrin method.

Histochemical observations on cholinesterase of regenerating limbs

A considerable amount of adult tissue of the stump was removed with the regenerate and served as the normal control. The stump tissues always showed a marked positive reaction for all cholinesterases (Plate, fig. A) and for specific cholinesterase (Plate, fig. B). Most of the positive reaction was localized in muscle which stained a deep golden brown and showed distinct striations. The nerve was also stained but much more faintly. In the control sections previously treated with a high concentration of DFP the reaction was entirely absent (Plate, fig. D).

We detected no reaction for any of the cholinesterases in the early, medium, and late regenerate buds (Plate, figs. A, B). The regenerate area was negative whereas the stump was strikingly stained. In the late palette stage a faint reaction occurred near the base of the regenerate where muscle first differentiates. It may be noted here that there are two types of muscle formed in the regenerate of the adult limb. The first is of muscle transected during amputation. This muscle undergoes sarcolysis at its end and releases many cells which extend in a stream into the wound area. During the palette stage cells of this stream begin to differentiate into muscle fibres; and these fibres give the first cholinesterase reaction. The second type of muscle is formed within the regenerate without morphological connexion to previous muscle and represents muscle removed completely during amputation. It appears later than the first type and is visible in the very early digital phase as sarcoblasts associated with delicate cross-striated fibrillae. By the time of the differentiation of this muscle, the specific cholinesterase reaction of the regenerate was almost as intense as that for the stump (Plate, fig. C); an adjacent control section treated with a high concentration of

DFP is shown in the Plate, fig. D. Nerve trunks of the regenerate, in contrast to muscle, stained very lightly. In summary, the results of histochemical study support those of the enzymological assays, namely that cholinesterase activity is quite low or relatively absent in the early stages of growth; it then increases sharply during the time of differentiation. The histochemical observations also show that the major part of the activity is located in the muscle.

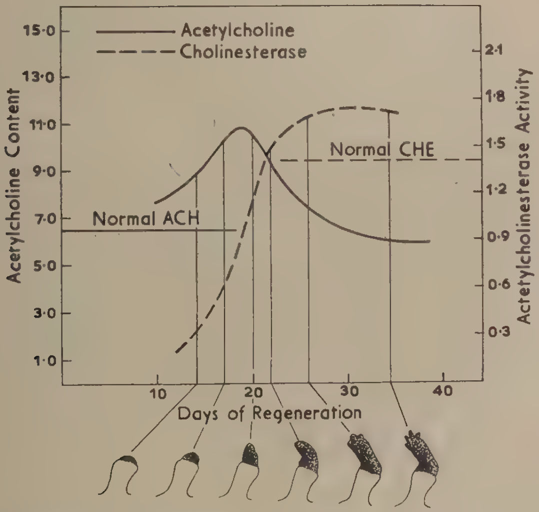
DISCUSSION

The results show that the activity of acetylcholinesterase is low during the phases of formation, accumulation, and rapid growth of the regenerate. It rises sharply during morphogenesis and histogenesis when it finally approaches (and may even exceed) the activity of esterase in the adult forelimb. The enzymological assay is supported by the histochemical one.

In a preceding paper (Singer, 1959*c*) the acetylcholine content of the regenerate was shown to be relatively high in the early stages of regeneration. Moreover, during the phases when mesenchymatous cells first appear among the distal wound tissues and accumulate to form the blastema, as well as when the blastema grows rapidly, the acetylcholine content rises high above that in non-regenerating tissues. Later, at the time of morphogenesis and histogenesis, acetylcholine declines and approaches that of the normal.

A good reason for the rise and decline of acetylcholine emerges from the present results. In Text-fig. 4 the cholinesterase activity and the acetylcholine content of the regenerate, scaled for direct comparison, are plotted for different stages of development. The graph shows an apparently causal relation between the acetylcholine-splitting enzyme and the acetylcholine content. When the activity of the enzyme is low, the rate of hydrolysis of acetylcholine is also low and the substance accumulates in higher quantities than in adult tissues. As the enzyme activity increases substantially the amount of acetylcholine per unit of regenerate declines until it finally approaches the normal value at the time of maximum cholinesterase content. There may also be other reasons for the accumulation of acetylcholine within the early growth, such as great abundance of nerve fibres in the regenerating tissue and greater choline acetylase activity.

The high level of acetylcholine and the low activity of cholinesterase occur during the phases of regeneration when the influence of the nerve upon the growth is most dramatic. It is worth recounting at this time the salient facts of this influence. If the adult stump is denervated at the time of amputation, mesenchymatous cells of regeneration do not appear (reviewed by Singer, 1952). If the regenerate is denervated later, during the phase of blastema formation, then, except for histogenesis of the individual tissues in the wound region, there is no further enlargement and regeneration of the missing parts (Singer & Craven, 1948). Once the regenerate bud has grown and contains many cells it will rearrange its substance and elongate to form a small limb in the absence of the nerve. Consequently, the phases of cellular appearance and accumulation and of subsequent rapid growth require the presence of the nerve; and it is during these phases that the cholinesterase activity is lowest and the content of acetylcholine is highest.



TEXT-FIG. 4. Comparison of acetylcholine content and acetylcholinesterase activity in regenerating forelimbs of various ages and stages (see legend for Fig. 3 of this paper; and for Fig. 2, Singer, 1959*c*; also Singer, 1959*a*).

The question must now be asked: is acetylcholine the agent of the nerve in its important action in regeneration of a body part? The causal relation between the acetylcholine amount within the growth and the activity of cholinesterase, which ensures a high level of the substance during the time when regeneration is most dependent upon the nerve, suggests an affirmative answer to this question. Such a conclusion seems to be supported further by the fact that atropine and other drugs which interfere with acetylcholine activity also suppress regeneration (Singer, Davis, & Scheuing, 1960). An affirmative conclusion would mean that the acetylcholine mechanism of the nerve, which is important in transmission or conduction of the nerve impulse, also serves as the 'trophic' mechanism. However, the variation in acetylcholine may be merely coincidental to the phases of regeneration most dependent upon the nerve; or it may be of other significance for regeneration. Indeed, in the light of information now emerging in our laboratory it is questionable that acetylcholine is the nervous agent of growth. This information is summarized in a review presented before the Growth Society (Singer, 1959*b*).

There are other possible actions of acetylcholine than that of a 'trophic agent'. It is said to be a surface agent which alters the permeability of protoplasmic membranes (Welsh & Taub, 1948). In this way it may function in regeneration by providing for the passage of important substances into cells. Another thought is that acetylcholine may serve as an important metabolic agent (Gerard, 1950) and indeed as a coenzyme (Welsh, 1948). In experiments from our laboratory (for preliminary summary see Singer, 1959*b*), we have observed regeneration under circumstances to which the variations in acetylcholine content could not be correlated. It is, therefore, conceivable that acetylcholine has no developmental significance; it may become of functional value only when anatomical structure is differentiated.

Aside from its possible interest in regeneration studies, it is of some importance and interest to compare the cholinesterase content of the developing regenerate with that of the embryo. A number of enzymological and histochemical studies have been made on cholinesterase activity in the amphibian embryo (Sawyer, 1943 *a*, *b*, 1955; Boell, 1948; Boell & Shen, 1944, 1949, 1950; Boell, Greenfield, & Shen, 1955). Cholinesterase appears in significant quantity at the time of first motility when the neuromuscular apparatus is differentiating. It is found at first in the spinal cord in a region where the early reflexes are initiated, and it appears thence (in order) in the hindbrain, midbrain, and forebrain. Therefore, during the early formative stages of embryonic development, there is little activity of the esterase. Later, associated with functional differentiation, there is a rise in cholinesterase which is much greater than the increase in some other enzyme systems or in the protein content of the nervous tissues (Boell, Greenberg, & Shen, 1955).

In the development of cholinesterase activity the regenerate resembles the embryo. The cholinesterase activity is low in the early formative stages of regrowth during the periods of so-called dedifferentiation and of cellular accumulation. It climbs slowly during the subsequent phase of rapid cellular multiplication and regenerate enlargement. At about the time of onset of morphogenesis, the activity rises sharply, and then approaches the value of adult tissues during the stage of digit formation. The sharpest rate of increase coincides with the time of early myogenesis. Thus, in the regenerate as in the embryo, the greatest increase in the activity of cholinesterase is correlated directly with the differentiation of muscle and therefore with the first signs of motility.

It seems likely that what little cholinesterase activity exists in the early stages is confined to the nerve fibres, and that the mesenchymatous cells of regeneration which constitute the blastema are relatively devoid of the active enzyme. Some of these cells are said to arise by a process of morphological dedifferentiation from muscle; assuming this to be the case, then dedifferentiation affects the activity of this enzyme in addition to degrading morphological structure. Later, at the time of histogenesis, differentiation or the reactivation of the enzyme occurs. Boell, Greenfield, & Shen (1955) showed that the development of enzyme activity during embryogenesis was about 10 times that of protein and consequently much more rapid than the development of structure. Protein determinations have not been made for the regenerate, but a rough comparison of the increase in dry weight and in cholinesterase activity suggests that a similar relation obtains for the regenerate. The increase in cholinesterase activity proceeds at a greater rate than increase in substance of the regenerate, especially during the period preceding and accompanying functional maturation and differentiation of muscle elements.

Although the development of cholinesterase activity in the regenerate resembles that in the embryo, it also differs from it. In the early premotile stages of *Amblystoma* (stage 36 of Harrison) cholinesterase activity is too low to be detected. It then appears suddenly and increases rapidly, coinciding with the first signs of motility. In the case of the regenerate, on the other hand, there is a detectable amount of activity in the earliest stage measured, namely, at a time when the blastema is just being formed. Consequently, regenerative—unlike embryonic—development occurs presumably from the start in the presence of some cholinesterase. The location of the enzyme is not known for certain at the moment but preliminary experiments on denervated regenerates seem to point to the nerve fibres themselves as the source of the activity. Nerve fibres appear among wound tissues within 2 or 3 days after amputation. They multiply rapidly and invade all tissues of the regenerate during subsequent days of development (Singer, 1949). Therefore, in the regenerate, detectable cholinesterase activity precedes by many days and stages of growth the first signs of motility and muscle differentiation, although the greatest rate of increase does coincide with functional and structural differentiation. The small amount of cholinesterase of the early regenerate is apparently not a prerequisite for later differentiation. Even after denervation of the regenerate and, therefore, after the loss of nerve cholinesterase, differentiation can occur only provided that the regenerate is of an adequate size at the time of nerve loss (Singer, 1952).

SUMMARY

1. Acetylcholinesterase activity in the regenerating forelimb of the adult newt, *Triturus*, was measured during various stages of development with the Hestrin colorimetric and the Warburg manometric method.

2. The activity of the enzyme is low during the early formative phases of development. It then increases, the greatest rate of change being in the stage just preceding that of differentiation of muscle. At the time of muscle differentiation the activity levels off and then returns to normal as regeneration draws to a close.

3. The variation in cholinesterase activity correlates well with the cycle of acetylcholine production during regeneration. The acetylcholine content is very high during early regeneration but then drops to a near normal level as esterase activity rises.

4. Since the nerve is required for the regeneration process and since the nerve

effect is most dramatic when the acetylcholine content is very high, the possibility that acetylcholine may be the agent of the nerve influence is assessed.

5. The enzymatic activity during regeneration is compared to that observed in embryonic development.

RÉSUMÉ

L'activité acétylcholinestérasique dans la régénération du membre antérieur du Triton adulte, Triturus

1. L'activité acétylcholinestérasique au cours de la régénération du membre antérieur du Triton adulte, *Triturus*, a été mesurée à différents stades du développement à l'aide de la méthode colorimétrique d'Hestrin et de la méthode manométrique de Warburg.

2. L'activité de l'enzyme est faible pendant les premières phases du développement. Elle augmente ensuite, le taux le plus élevé étant au stade qui précède immédiatement la différenciation du muscle. Au moment de la différenciation du muscle, elle cesse d'augmenter et revient à l'activité normale quand la régénération touche à sa fin.

3. La variation de l'activité cholinestérasique est en corrélation avec le cycle de la production d'acétylcholine pendant la régénération. Le contenu en acétylcholine est très élevé pendant le début de la régénération mais ensuite il tombe à un niveau presque normal quand l'activité estérasique s'élève.

4. Comme le système nerveux est nécessaire au processus de régénération et que l'effet du nerf est le plus marqué quand la teneur en acétylcholine est la plus élevée, l'hypothèse suivant laquelle l'acétylcholine est l'agent de l'influence nerveuse est discutée.

5. L'activité enzymatique durant la régénération est comparée à celle que l'on observe dans le développement embryonnaire.

ACKNOWLEDGEMENTS

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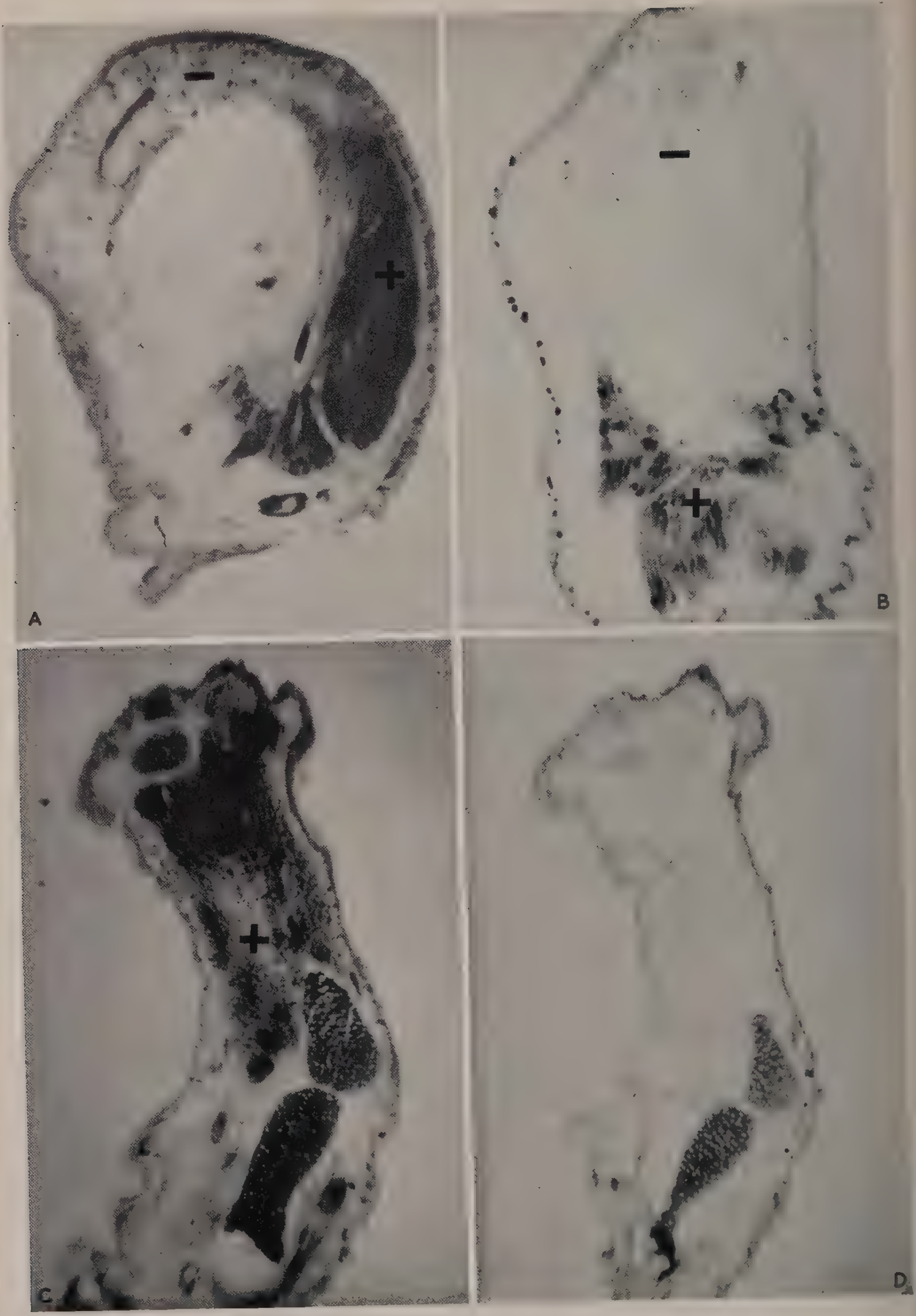

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EXPLANATION OF PLATE

FIGS. A–D. Sections of regenerates of various ages tested histochemically for cholinesterase activity.

FIG. A. Early bud and amputation stump showing distribution of 'all' cholinesterases. The reaction is negative (light staining) in the regenerating tissues (−) but highly positive (dark staining) in muscles of stump (+). Nerves show faint positive reactions. The central cavity is the space normally occupied by the humerus. ×35.

FIG. B. Very late bud or early palette and amputation stump showing distribution of specific acetylcholinesterase. There is relatively little reaction (−) in the entire regenerate area; but a strong positive reaction (+) in the stump. ×27.

FIG. C. Digital regenerate showing specific acetylcholinesterase. Note the strong reaction (+) among the muscles of the regenerate. ×21.

FIG. D. Section adjacent to that of fig. C. Section treated with di-isopropylfluorophosphate (DFP) (10^{-3} M), then tested for acetylcholinesterase. Note complete absence of reaction in regenerate and stump. ×21.

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Influence of the Supra-oesophageal Ganglion on Posterior Regeneration in *Nereis diversicolor*

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INTRODUCTION

HUBL (1956) demonstrated that if the supra-oesophageal ganglion of lumbricid oligochaetes is removed at the time of amputation of a number of posterior segments, caudal regeneration is totally inhibited, but if the ganglion is not removed until 24–48 hours after amputation of the posterior segments, regeneration proceeds at the normal rate. A comparable phenomenon may occur in nereid polychaetes. Removal of the prostomium (Casanova, 1955) or, more precisely, of the supra-oesophageal ganglion (Durchon, 1956), retards regeneration but does not prevent it, but as these experiments were performed on the day following amputation of the posterior segments it is still not certain whether the brain of *Nereis* is, at any stage, essential for posterior regeneration as it clearly is in lumbricids.

In his investigation of *Lumbricus* and *Allolobophora*, Hubl (1956) found that neurosecretory cells in the supra-oesophageal ganglion, the *b*-cells, showed intense secretory activity as soon as the worm was injured and the ventral nerve-cord damaged. In the polychaete *Nephtys*, too, certain neurosecretory cells in the supra-oesophageal ganglion show signs of secretory activity within 6 hours of the loss of posterior segments (Clark & Clark, 1959). There is thus a very prompt response in the supra-oesophageal ganglion of lumbricid oligochaetes and nephtyid polychaetes to the loss of segments, and it is possible that this is true of nereids also. In the experiments performed on nereids by Casanova and Durchon, the supra-oesophageal ganglion remained intact for 24 hours after the segments had been removed, i.e. during the period when the initial response in the ganglion might be expected to occur. The experiment described below has been designed to complement those of Casanova and Durchon and to investigate the role of the supra-oesophageal ganglion of *Nereis* during the initial stages of regeneration.

MATERIALS AND METHODS

It has been necessary to select a suitable species of *Nereis* for this experiment with some care because removal of the supra-oesophageal ganglion causes pre-

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cocious metamorphosis and maturation of the gametes in many species. (Durchon, 1952; Hauenschild, 1956 *a*, *b*) and it is not known to what extent the profound morphological reorganization that accompanies metamorphosis may interfere with regeneration. The females of *N. diversicolor*, which were chosen, have the following advantages.

1. This species does not form a heteronereis in the natural state (e.g. Dales, 1950), nor when the supra-oesophageal ganglion is removed (Durchon, 1952). 2. The effect of brain removal on oocytes is much less than that on spermatocytes in *Nereis* (Durchon, 1952; Hauenschild, 1956*b*). 3. Females form a majority of most populations of *N. diversicolor* (Dales, 1950) and so are readily available.

The animals were anaesthetized in isotonic magnesium chloride solution and decerebrated under sterile conditions. The supra-oesophageal ganglion can be removed rapidly and with little loss of blood by making incisions in the prostomium in front of the anterior eyes, behind the posterior eyes, and along the side, lifting the flap of epidermis and picking out the encapsulated ganglion with forceps. The worms were kept in individual dishes and given several changes of sterile water until they had recovered from the anaesthetic.

A number of posterior segments, amounting to one-fifth of the total length of the worm, was removed without anaesthesia by pinching with forceps; this generally causes the worms to autotomize.

Both intact and decerebrated worms were kept in glass aquaria containing sea-water diluted with tap-water in the proportion of 3 : 1, and lengths of glass tubing of appropriate dimensions were provided for the worms to live in. All the animals were starved because decerebrated worms are unable to feed.

At the end of the experiment all worms were fixed in Bouin's fluid. Paraffin sections of the regenerating tip were stained with haematoxylin and eosin, and sections of the prostomium of regenerating worms and of controls with paraldehyde fuchsin, haematoxylin, and picro-indigocarmine (Gabe, 1953).

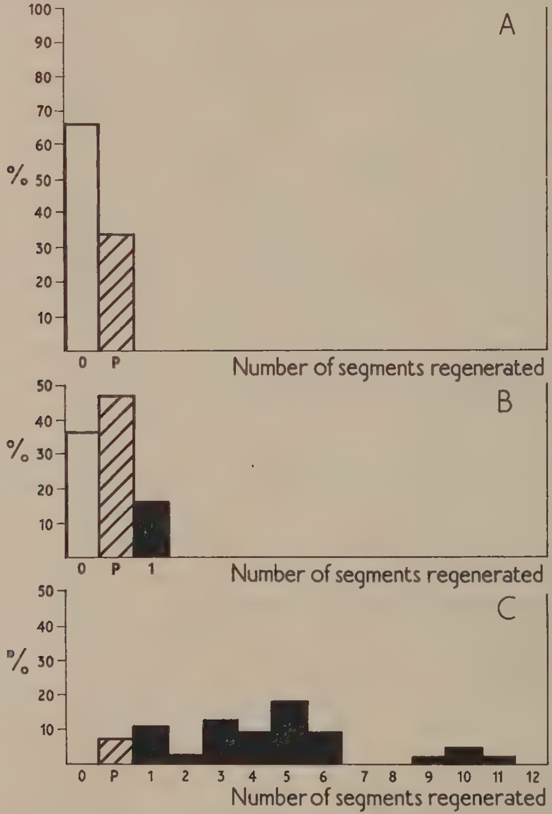
RESULTS

Experimental

Specimens of *N. diversicolor* were divided into three lots and treated as follows: group 1 was decerebrated by the technique described above, and the posterior segments were removed 24 hours later (21 specimens survived); the animals of group 2 had their posterior segments removed (or else they had broken off accidentally during collecting) and the worms were decerebrated 72 hours later (19 survived); the animals of the control group had their posterior segments removed, but the supra-oesophageal ganglion was left intact (47 survived).

Each group of worms was maintained at a temperature of 15 ± 2° C. for 30 days and the specimens were then fixed and examined. Mortality among the controls was less than 11 per cent. and among the experimental animals 20 per cent.; most deaths occurred during the final week of the experiment.

Controls (Text-fig. 1c). In the course of the experiment 11 worms died. Twelve more failed to undergo regeneration, but instead the posterior part of the body became necrotic and the animals sluggish and unhealthy. Had the experiment continued for another week, most if not all of these worms would have died also, and they will be omitted from further consideration. The reason for this mortality is unknown, the more so since necrosis of the posterior end was not observed among any of the experimental animals. Of the remainder, 32 worms began to proliferate new segments (average 4·3 segments) and 3 worms regenerated only a pygidium. Histological examination of the regenerating tip of the latter worms revealed considerable mitotic activity in the terminal part of



TEXT-FIG. 1. Number of segments regenerated by *N. diversicolor* in 30 days. A, supra-oesophageal ganglion removed one day before posterior segments amputated; B, supra-oesophageal ganglion removed 3 days after posterior segments amputated; C, controls, supra-oesophageal ganglion intact. O, no regeneration; P, pygidium only regenerated.

the intestine and in the epidermis beneath the nerve-cord near the pygidium. In a more detailed investigation of regeneration in the related polychaete *Nephtys*, we have found these to be the sites of the greatest mitotic activity at the onset of segment proliferation, and it may be inferred that these three specimens of *Nereis* were in the process of segmentation, although no signs of it were visible macroscopically.

Group 1 (Text-fig. 1A). None of the worms that were decerebrated before amputation of the posterior region regenerated new segments. Seven specimens regenerated a pygidium with anal cirri, but no mitotic activity could be detected in the posterior segments by histological examination. The majority of these worms did not proceed beyond the stage of wound healing.

Group 2 (Text-fig. 1B). Three of the worms that were decerebrated after the loss of the posterior segments regenerated a single new segment. Nine specimens regenerated a new pygidium and the rest did not regenerate at all. Histological examination of those that had regenerated a pygidium revealed only a few mitotic figures in the cells of the posterior part of the gut in two specimens, and no mitotic activity in the others.

Histological examination of the brain

Specimens of *N. diversicolor* were fixed at various intervals after the posterior segments had been removed. The neurosecretory activity of the supra-oesophageal ganglion of each experimental animal was compared with that of the brains of worms that were not regenerating and also with worms that were already regenerating new segments when they were collected. The types of neurosecretory cells in the brain of *Nereis*, their distribution in the ganglion, their cytology and staining reactions are all well known (Scharrer, 1936, 1937; Schaefer, 1939; Bobin & Durchon, 1953; Gabe, 1954). Our preparations were stained with paraldehyde fuchsin, which is known to stain the secretory products of all four types of neurosecretory cell in the brain of *Nereis* (Gabe, 1954).

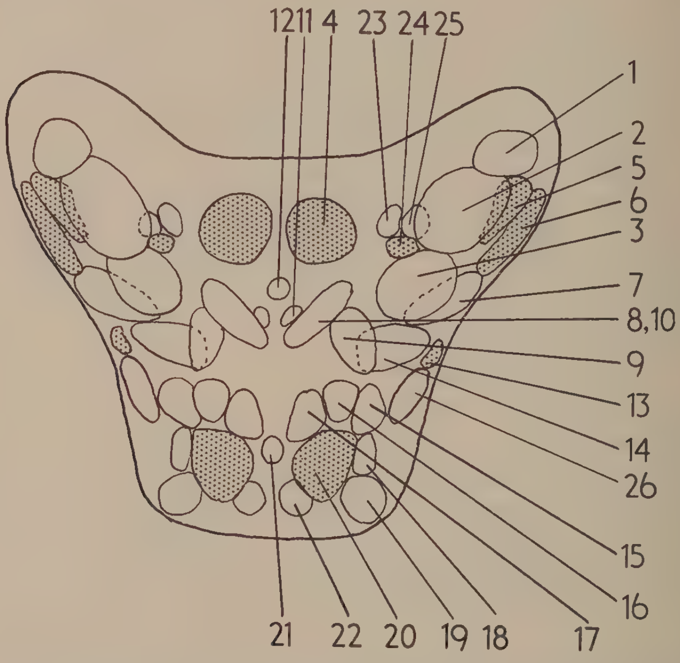
TABLE 1

Neurosecretory activity in the supra-oesophageal ganglion of intact and regenerating Nereis

Ganglionic nucleus	*Control*	*Time after amputating posterior segments*								R_1	R_2
		3 h	*6 h*	*12 h*	*1 day*	*2 days*	*3 days*	*4 days*	*5 days*		
20	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
24	++	++	++	++	++	++	++	++	++	++	++
13	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	++	++	++	++	++	+(+)	+	+
5	?	?	+	+	+	−	+	+	+	+	?
6	?	?	+	+	+	+		+	+	+	0

Neurosecretory activity estimated subjectively on the basis of the number of cells containing neurosecretory material and the quantity of secretion. R_1 and R_2 are worms found to be regenerating when collected and had proliferated 15–20 new segments.

The result of the examination of the supra-oesophageal ganglion of intact and regenerating worms is given in Table 1. The nomenclature of the ganglionic nuclei follows that proposed by Holmgren (1916) in his account of the anatomy of the brain of *N. diversicolor*. We detected neurosecretory material in the cells of six pairs of ganglionic nuclei (Text-fig. 2).



TEXT-FIG. 2. Position of the ganglionic nuclei in the brain of *N. diversicolor* (diagrammatic). (Based, in part, on Holmgren, 1916.) Stippled nuclei are those in which neurosecretory cells were observed.

The densest accumulations of secretion were found in nuclei 20, 24, and 13 in all the worms that were examined. These nuclei present the same appearance in regenerating as in intact animals.

Both the quantity of secretory material and the number of cells containing granules of secretion in nucleus 4 were greater in the ganglia of worms fixed 12 hours or more after amputation of the posterior segments than in the controls. Possibly there is less secretion in this nucleus in the brain of worms fixed 5 days after amputation of the posterior segments, and certainly in worms that had regenerated 15–20 segments the cells in this nucleus showed no greater activity than the corresponding cells in the controls.

Nuclei 5 and 6 are contiguous and cannot be separated with confidence. Rather more cells in them contain secretory material in worms fixed 6 hours or more after the posterior segments had been amputated than in the controls, but the difference is slight.

DISCUSSION

It is clear that *N. diversicolor* resembles the lumbricids in one respect: regeneration of new segments is inhibited if the supra-oesophageal ganglion has been extirpated before the posterior segments are amputated. Extirpation of the ganglion after the segments have been removed retards regeneration in *Nereis*, but has no effect on posterior regeneration in lumbricids (Hubl, 1956). It has been apparent for some time that the supra-oesophageal ganglion of both groups of worms is a source of hormones that play some part in regeneration (Harms, 1948; Hubl, 1953, 1956) and it is evident that the cerebral 'regeneration' hormones are essential during the initial phase of regeneration, and that they are of diminishing importance thereafter. The period immediately following the loss of segments is marked by changes in the neurosecretory activity in the supra-oesophageal ganglion. In lumbricids, numerous *b*-cells begin to secrete (Hubl, 1956), in *Nephtys*, cells in nucleus *S* produce detectable quantities of neurosecretory material within 6 hours and in nuclei *Z* 24–48 hours after the loss of posterior segments (Clark & Clark, 1959). In *Nereis* there is an increase in secretory activity in the cells of nuclei 4, 5, and 6 within 12 hours of injury to the worm, but the change is by no means as clear-cut and dramatic as in the other worms.

SUMMARY

1. Extirpation of the supra-oesophageal ganglion of *N. diversicolor* before the removal of a number of posterior segments inhibits the formation of new segments. At most, a new pygidium is regenerated.

2. Extirpation of the supra-oesophageal ganglion 3 days after the removal of a number of posterior segments retards but does not inhibit segment-formation.

3. An increase in the quantity of neurosecretory material produced by cells in certain nuclei of the brain of regenerating worms becomes detectable about 12 hours after the posterior segments have been removed.

4. *Nereis* resembles lumbricid oligochaetes in that the supra-oesophageal ganglion plays an essential role during the initial phases of regeneration, but that once regeneration is initiated, its importance declines.

RÉSUMÉ

1. L'extirpation du ganglion supra-œsophagien de *Nereis diversicolor*, avant l'ablation d'un certain nombre de segments postérieurs, empêche la formation de nouveaux segments. Tout au plus un nouveau pygidium est régénéré.

2. L'extirpation du ganglion supra-œsophagien, trois jours après l'ablation d'un certain nombre de segments postérieurs, retarde mais n'empêche pas la formation de segments.

3. Un accroissement de la quantité de matériel neuro-sécrétoire est produit par les cellules de certains noyaux cérébraux des vers en voie de régénération. Cette augmentation peut être décelée environ 12 heures après que les segments postérieurs ont été excisés.

4. *Nereis* évoque les oligochètes lumbricides parce que le ganglion supra-œsophagien joue un rôle essentiel pendant les phases initiales de la régénération, mais une fois la régénération commencée, son importance diminue.

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Aberrant Nerve Fibres in Human Development

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WITH FOUR PLATES

INTRODUCTION

IN the early years of this century debate concerning the development of nerve fibres became more intense. During the previous decade, following the developmental studies of His (1883, 1886) and the early embryological studies of Ramon y Cajal (1890), the neurone theory as proclaimed by Waldeyer in 1891 seemed assured of victory; but when, with Apáthy and Bethe, new technical developments diverted attention from the whole neurone to its apparent constituents, the neurofibrillae, the simple concept of the outgrowth of the nerve fibre became enmeshed in complexity. Methods for their impregnation with silver were soon elaborated (Bielschowsky, 1904; Ramon y Cajal, 1903), and Held (1907) affirmed that a network of neurofibrillae preceded the appearance of the definitive nerve process. This claim became associated with the much older views of Hensen (1864, 1876) that protoplasmic strands were the forerunners of the nerve fibres and constituted a ground plan for the later development of the peripheral nervous system.

In 1908 Ramon y Cajal described a variety of observations on neurogenesis in the chick embryo which were inconsistent with this theory. Among them were some instances of aberrant fibres within the spinal cord and medulla at 4 days of incubation. These *axones égarées* included examples which penetrated the central canal; some of these re-entered the cord at another level. Others at first grew outwards in the normal way towards the surface of the cord but before reaching it turned sharply in the opposite direction and ended in a giant growth-cone pointing inwards. Ramon y Cajal concluded that these abnormalities showed that in general the growth-cone possessed 'une liberté de mouvement et d'allure qui ne concilie pas facilement avec la supposition d'un système de liens interneuronaux . . . ni avec celle d'une charpente de voies préétablies par lesquelles les fibres nerveuses jeunes seraient obligées de marcher'.

In more recent years several papers have been concerned with developing nerve fibres which follow an abnormal course, both within neuroepithelia and in the peripheral nervous system. In the developing inner ear, Tschernjachiwsky

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(1929) found that within the utriculus of a 3-month human foetus there were fibres which entered the lumen and which for some way grew freely into the fluid within. Not always, however, do the *fibres égarées* lose their way in this manner. In a further study of the auditory complex of nerves in the human foetus, Shute (1951) found that while some neurites were of this kind, others could 'reach their destination by an abnormal or devious route'. Nor are aberrant fibres necessarily rare abnormalities. Bremer (1921) found that in 90 per cent. of human embryos at the 18-mm. stage, transient and recurrent branches of the abducens nerve of no apparent morphological significance run caudally to a number of muscles, both branchial and somitic.

In the present paper the study of *fibres égarées* in the human foetus is continued both in further observations on the developing abducens nerve and also in a study of such fibres within two rudimentary epithelial structures which are innervated at an abnormally early stage. These particular observations continue an inquiry begun by Ramon y Cajal himself in 1919 in a study of the penetration of nerve fibres into epithelia, the detailed course of which, he found, includes a phase of exploratory and transient elements.

Aberrant branches of the VIth cranial nerve in human embryos and foetuses

The presence of recurrent branches arising in the course of the cranially directed abducens nerve has been recorded by a number of investigators for a number of vertebrates. Bremer (1921) summarized the literature on such fibres and added a detailed account of his own findings in a number of human embryos. He states that the recurrent fibres are infrequently to be found in *Acanthias, Heptanchus,* and in the pig, and that they are absent in the chick, the lizard, the sheep, and the rabbit. According to Bremer they are most commonly present in Man, 'where they appear in about 90 per cent. of embryos up to 18 mm. and less frequently up to 31 mm., since they may begin to degenerate at 15 mm.' Bremer's material included no sections of embryos in which the peripheral nervous system had been specifically stained. All our silver impregnated (de Castro, Ranson, Bodian) human embryos possess the recurrent fibres and they are occasionally found in the younger foetal stages; we can also record their presence in equivalently treated rabbit embryos though, in this species, they are much fewer in number than in human specimens.

The recurrent fibres pass caudally to end, for the most part, in the mesenchyme surrounding the ventro-lateral aspect of the hindbrain. In a number of the specimens, however, some of the recurrent fibres unite to form a distinct nerve bundle which passes to the region of the glossopharyngeal nerve, at the level of its superior ganglion, with which it may unite; in two specimens the aberrant abducens fibres could be traced beyond the IXth nerve to join the vagus and accessory trunks. So far as we can determine these recurrent fibres disappear completely in later human development. When our findings are taken in conjunction with Bremer's account, however, it is apparent that in a number of vertebrates there is a transient distribution of nerve fibres, which make their exit from the central nervous system in association with the VIth nerve to regions of the embryo which cannot readily be considered as morphologically appropriate for this nerve.

There is another aberrant distribution of VIth-nerve fibres which Bremer and, so far as we can determine, other investigators have missed, probably because silver impregnation techniques were not used in the preparation of the embryonic material studied. When the VIth nerves are followed towards the primordia of the eye muscles in adequately impregnated human embryos of 15–20 mm. C.R. length, a number of fibres are seen to leave the main trunks of the nerves and to pass towards the anterior extremity of the notochord. When cartilage commences to appear in this region these medially directed aberrant fibres are caught up in the chondrification process and come to be included within the developing matrix of the basisphenoid cartilages (Plate 1, figs. 1, 2). There they can be found, like flies in amber, until about the 60-mm. stage, after which they apparently disappear; at any rate, they cannot be identified in our older foetuses, though the impregnation of the cranial nerves is generally very good.

In the earlier stages, as they are being caught up in the process of chondrification, the aberrant VIth-nerve fibres provide a remarkable picture (Plate 1, figs. 3, 4). They are usually heavily impregnated with reduced silver and the individual fibres present an appearance of wire-like rigidity. No Schwann cells can be found on them. A single fibre frequently possesses complex angulations in its course, the microscopic picture presumably reflecting the constrictive influence on the fibre of the developing cartilage matrix. In such stages the endings of the aberrant fibres in the cartilage can frequently be identified by the possession of terminal bulbs. As development proceeds these bulbs disappear and the aberrant fibres end in an indefinite network in which the actual terminations can only dubiously be identified. Finally, in those stages (48-mm. and 62-mm. foetuses; Plate 1, fig. 5) at which the aberrant fibres can last be identified, the fibres would seem to have fragmented into separate lengths, for it is often impossible to trace continuity between fibres in the same or in adjacent sections. In yet older specimens no trace of the aberrant fibres in the basisphenoid region is to be found.

The distribution of the aberrant fibres to the basisphenoid region is, therefore, a transient one. Nevertheless, they are, it would seem, constantly present from the 12-mm. stage until the 60-mm. stage, that is to say, from about the 38th to the 90th day of development. Why they should grow into the mesodermal tissue surrounding the cranial extremity of the notochord is not apparent. Hughes & Tschumi (1960), however, have observed in grafting experiments in *Xenopus* what may be an attractive influence of notochord on motor nerve fibres. Whatever the explanation for the anomalous course and termination of the aberrant fibres, they become caught up in the chondrification of the basisphenoid. In this fact may lie the explanation for their persistence. No macrophages have been observed along the course of the disappearing fibres. The inability of such scavenging cells to enter the cartilage matrix could explain the anatomical persistence of the aberrant fibres which may, in fact, have ceased to be living elements for some time before they disappear. We are, of course, unable to comment effectively on the behaviour of the abducens nerve-cells which give origin to the aberrant fibres. They may degenerate with the disappearance of the fibres, or the latter themselves may merely be, or become, collateral branches of nerve fibres which proceed forward to aid in the innervation of the lateral rectus muscle.

Nerve fibres in Chievitz's organ

In his account of the development of the salivary glands Chievitz (1885) described an epithelial strand which, in the 10-week human foetus, extends backwards in the mesoderm, parallel to the lateral border of the buccal sulcus, on the medial side of the mandible, and in close relation to the medial pterygoid muscle. In the intervening period attention has been drawn to the structure by a number of other investigators who have used such different names for it as the *ramus mandibularis ductus parotidei* (Weishaupt, 1911), the *orbital inclusion* (Schulte, 1913), *Chievitz's organ* (Broman, 1916), and the *tractus bucco-pharyngeus* (Brachet, 1919). It has been described in embryos of a number of mammalian species; Fahrenholz (1937) indicates that it has been found in Man, dog, horse, pig, cow, sheep, musk-deer, fallow-deer, hedgehog, mole, *Tupaia*, mouse, rat, guinea-pig, rabbit, *Leptonychotes*, *Vespertilio*, and opossum. It is, however, possible that different epithelial structures have been included within the descriptions, whatever general term is used for the strand. Further, the precise nature and significance of the epithelial band has by no means been established. It has been variously interpreted as a vestigial salivary gland (Broman, 1916), an organ of internal secretion (Schulte, 1913), a structure of undetermined nature (Moral, 1913), or as merely an epithelial strand of no morphological or functional import, which becomes separated from the cheek in the course of the developmental mechanical changes in the region. The last interpretation, which was put forward by Brachet (1919) and supported by Bollea (1924) and Casarotto (1928), is the one which seems currently to be the most acceptable and to which our own observations incline us.

Our present concern, however, is not to discuss the nature and significance of Chievitz's organ itself but to draw attention to the remarkable association it possesses with branches of the buccal branch of the mandibular ramus of the trigeminal nerve. The association was briefly commented on by Brachet (1919); it is well shown in a 43-mm. human embryo (Plate 2, figs. 6, 7, 8) impregnated with de Castro's modification of Cajal's silver impregnation technique. In this specimen Chievitz's organ has lost its connexion with the buccal epithelium, and it extends backwards on the medial side of the mandible as an elongated cord of compactly arranged epithelial cells. The buccal branch of the mandibular nerve can be traced in its course to the cheek lateral to the epithelial strand. As the branch passes the strand, a bundle of nerve fibres leaves it and passes along the surface of the epithelial cells. Individual fibres enter the strand and terminate in the interstices between its constituent cells. At this time, it must be stressed, the adjacent buccal epithelium possesses no innervation (Plate 2, fig. 9).

The nerve fibres which enter Chievitz's organ do not seem to possess neurilemmal sheaths. They are, of course, non-myelinated. Their method of termination is variable, some of them ending by becoming increasingly attenuated until they can no longer be resolved with light microscopy. Other fibres, however, possess definite end-bulbs. The richness of the nerve supply is quite remarkable, for it exceeds considerably that which is ever found, with the same impregnation techniques, in the epithelium of the buccal mucosa or of the cheek skin. Further, the innervation of Chievitz's organ is of quite a different order from that of the salivary glands, in the epithelium of which no nerve fibres have been observed in any of our foetal specimens.

Our material includes a number of embryos and foetuses from the 15-mm. to the 150-mm. C.R. length stages. A definite innervation of the epithelium of Chievitz's organ is first obvious in a 30-mm. embryo (Plate 3, fig. 10), and it appears to reach its maximum between the 40-mm. and 60-mm. stages. The nerve supply to the organ is distinctly reduced in a 92-mm. foetus in which a specific nerve bundle from the buccal branch of the mandibular trunk could no longer be identified. By the 150-mm. stage the number of nerve fibres in the organ has been very considerably reduced, and terminal bulbs can no longer be identified. A plexiform arrangement of nerve fibres round the epithelial cells is, however, still present (Plate 3, fig. 11). In none of our human material beyond the 150-mm. stage have we been able to identify Chievitz's organ; this material, however, is not available in uninterrupted series. Its presence in a full-time human foetus has been recorded by Ramsey (1935).

Nerve fibres in relation to the paramesonephric ducts in male human foetuses

Figs. 12 and 13 of Plate 3 are photomicrographs of sections through the urogenital cords of a male and a female human foetus. Each specimen had a C.R. length of 46 mm.; their general embryological state and the associated obstetrical histories also suggest that the two specimens are of equivalent developmental age. The sections illustrated show, however, that the genital ducts are differentiating; in the male the paramesonephric (Mullerian) ducts have fused to form an irregular epithelial cord, while in the female they form a well-established utero-vaginal canal. On the other hand, the mesonephric (Wolffian) ducts are well developed in the male and are commencing to retrogress in the female. In each sex the genital ducts are surrounded by mesodermal condensations which in the female will develop into the myometrium and in the male into the fibrous and muscular tissue of the prostate gland. Flanking the mesodermal condensations are the differentiating neuroblasts of the pelvic ganglia. In each sex, in appropriately impregnated material, nerve fibres can be traced from these ganglia into the mesodermal condensations. In the female, in later foetal stages, these nerve fibres become distributed to the muscular coats of the uterus and upper vagina. In male foetuses, however, a remarkable difference is found. Some of the nerve fibres are indeed distributed to the myoblasts which will become part of the sheath of the prostate gland. But many of them pass to the wall of the epithelial tube—the *uterus masculinus*—arising from the conjoined paramesonephric ducts. Some of these fibres terminate on or in relation to the surface of the epithelium; others enter the epithelium (Plate 3, fig. 14; Plate 4, figs. 16, 18), and in older specimens bundles of the nerve fibres may actually perforate through into the lumen, which develops in the *uterus masculinus*, and pass upwards and downwards inside this cavity (Plate 4, figs. 15, 17). The nerve fibres frequently possess bulbous terminals (Plate 4, figs. 18, 19) and in the older specimens considerable lengths of nerve fibre may show a remarkably swollen appearance (Plate 4, fig. 19). We have found this invasion by nerve fibres of the epithelium of the *uterus masculinus* in human foetuses of as late as the 150-mm. C.R. length stage. Our older male foetal material, which is sparse and not very satisfactory, does not show such nerve fibres, and the aberrant innervation does not appear to be present in postnatal specimens. In the period of development between the 40-mm. C.R. stage and the 150-mm. C.R. stage, however, a rich distribution of nerve fibres to the epithelium of the *uterus masculinus* is present in all of our available specimens. Such appearances are not presented by any of our female foetuses and, we can add, no equivalent appearance has been found in relation to the retrogressing mesonephric ducts in female specimens.

DISCUSSION

Three examples of atypical behaviour by nerve fibres in the course of human development have been described. Each of them raises the question of why the nerve fibres have taken their aberrant courses. We have tentatively suggested that for the abducens fibres the attraction may be exercised by the cranial end of the notochord. The long course of this nerve, its proximity to the notochord in this course, and the relative remoteness of the somitic material which it has to reach, may be the explanation of the limitation of the aberrant process to this nerve. For the aberrant fibres in the *uterus masculinus* and in Chievitz's organ the attraction presumably emanates from the epithelia of the two structures.

It was in his paper on the development of the vertebrate retina that Ramon y Cajal (1893) first advanced his hypothesis of 'neurotropism' in an attempt to account for the growth of a nerve fibre towards its destination, though he admitted the unlikelihood of any chemotropic stimulus influencing an elongating neurite across more than a relatively short distance. In the development of cutaneous nerves, however, the orientation of the fibres within the embryonic dermis and their manner of advance towards the overlying ectoderm appeared to him to provide clear instances of chemotropic attraction (Ramon y Cajal, 1919). He suggested that the embryonic dermis receives from the epithelium some 'enzyme' with a stimulatory action on the growth-cones of the advancing neurites.

To his description of the innervation of epithelia in late foetal and early post-natal life we can now add instances of the precocious entry of nerve fibres into two transitory organs belonging to different germ layers, at a time well before their period of dissolution, and when no adjacent epithelia have yet received any innervation. Whatever may be the factors which govern the entry of nerve fibres into epithelia in general, they must also operate, though prematurely, in these special instances.

Ramon y Cajal described four successive phases in the pattern of the invading nerve fibres in normal epithelia. In these prematurely innervated structures this sequence is apparently halted at the stage of irregular and exploratory neurites. In the *uterus masculinus* they become grossly enlarged and recall the *fibres égarées* which ended in giant end-bulbs described by Ramon y Cajal (1908) in the spinal cord of an embryo chick. The hypertrophy of the neurites within the *uterus masculinus* extends, however, to the whole terminal portion of the fibre, and is on a much larger scale.

Among his many observations of the growing nerves in the tail fins of tadpoles, Speidel (1933) described how the growth-cone of a fibre halted by an obstruction became enlarged. In the present instance, however, the undifferentiated epithelium of the *uterus masculinus* shows no features which might block the pathway of a growing fibre. Moreover, we see a wholly different reaction of neurites to mechanical constriction in the fibres of the abducens nerve trapped in the chondrifying basisphenoid; these take on a wire-like form with straight sections joined by sharp angles.

Heidenhain (1911) discussed the successive factors which may influence the gradual development of the peripheral nervous system and suggested that each organ receives an excess of nerve fibres, and that the final pattern of innervation is partly attained by the atrophy of superfluous elements. This must apply with especial force to organs in which the course of development is different in the two sexes. It seems reasonable to suppose that the paramesonephric duct in all embryos receives a total supply of neuroplasm adequate for the development of the basic pattern of innervation of a foetal uterus, but which in the male is excessive for the requirements of a developing prostatic utricle. In this way superfluous fibres may become engorged and break through the lumen of the organ with giant end-bulbs, as has here been described and illustrated.

Whatever the nature of the forces which govern the innervation of embryonic structures, it is clear that entering fibres are not merely passively accepted, but their growth and arrangement must be subject to a continuous control exercised in some way by the developing organ itself. Ramon y Cajal's neurotropism still remains the most plausible explanation of these influences, particularly since the demonstration by Hamburger and his colleagues (Hamburger, 1952) that the growth of nerve fibres is stimulated by specific substances. Although these experiments still lie outside the course of normal embryonic development and no directional effects have yet been discovered, there is now proof that nerve fibres can respond to chemical influences (Levi-Montalcini, 1958). It would be an interesting inquiry in foetal endocrinology to examine whether the nerve fibres of developing reproductive organs are in any way sensitive to the action of sexual hormones.

SUMMARY

1. Attention is drawn to the regular, though transient, presence of aberrant nerve fibres (*fibres égarées*) in three different regions of human embryos and foetuses.

2. One of these regions is the cartilaginous basisphenoid into which fibres from the abducens nerves pass. The other two regions are the epithelial cord, known as the organ of Chievitz, and, in male specimens, the epithelium of the fused paramesonephric ducts.

3. The possible causes for the atypical behaviour of the aberrant nerve fibres is discussed. It is tentatively suggested that the abducens nerve fibres may be attracted medially by some influence emanating from the cranial end of the notochord. For the aberrant fibres in the *uterus masculinus* and in Chievitz's organ the neurotropic attraction is presumed to emanate from the constituent epithelia of the two structures.

RÉSUMÉ

Sur des fibres nerveuses aberrantes dans le développement de l'homme

1. Les auteurs attirent l'attention sur la présence régulière, quoique passagère, de fibres nerveuses 'égarées' dans trois régions différentes des embryons et des fœtus humains.

2. Une de ces régions est le basisphénoïde cartilagineux dans lequel des fibres s'introduisent en provenance des nerfs efférents. Les deux autres régions sont le cordon épithélial, connu sous le nom d'organe de Chievitz, et, dans les spécimens mâles, l'épithélium des canaux paramésonéphriques fusionnés.

3. La cause possible du comportement atypique des fibres nerveuses égarées est discutée. On envisage l'hypothèse que les fibres nerveuses efférentes puissent être attirées en direction médiale par quelque influence émanée de l'extrémité craniale de la notochorde. Quant aux fibres aberrantes de l'utérus masculinus et de l'organe de Chievitz, elles pourraient être expliquées par l'attraction neurotropique qui émane des épithéliums constitutifs de ces deux structures.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Left abducens nerve (VI) and fibres from it which can be traced into the chondrifying basisphenoid in a 30-mm. human embryo (H. 180, de Castro technique). ×108.

Fig. 2. High-power view of the nerve fibres in the basisphenoid shown in fig. 1. ×684.

Fig. 3. Nerve fibres in left side of basisphenoid condensation in a 17·5-mm. human embryo (H. 191, de Castro technique). ×684.

Fig. 4. Nerve fibres in right side of basisphenoid condensation in 17·5-mm. human embryo (H. 191). ×684.

Fig. 5. Nerve fibres in basisphenoid cartilage in a 62-mm. human embryo (H. 209, de Castro technique). This is the oldest specimen in which such fibres have been found. ×684.

Plate 2

Fig. 6. Sagittal section through right Chievitz's organ (*C*) in a 43-mm. human foetus (H. 206, de Castro technique) to show branches of the long buccal nerve (*N*) passing to it. ×108.

Fig. 7. High-power view of portion of fig. 6 to show nerve bundle skirting Chievitz's organ and sending fibres amongst its epithelial cells. ×684.

Fig. 8. Nerve fibres in epithelium of left Chievitz's organ in 43-mm. human foetus (H. 206, de Castro technique). ×684.

Fig. 9. Transverse section through right Chievitz's organ (*C*) in a 30-mm. human embryo (H. 180, de Castro technique). Note nerve fibres entering epithelium of the organ and absence of such fibres in the parotid gland (*P*) and in the buccal epithelium (*B*). ×108.

Plate 3

Fig. 10. High-power view of section through right Chievitz's organ shown in Plate 2, fig. 9. ×684.

Fig. 11. High-power view of section through right Chievitz's organ in a 150-mm. human foetus (H. 177, de Castro technique). Nerve fibres are still present round the organ but are absent in the epithelium itself. ×684.

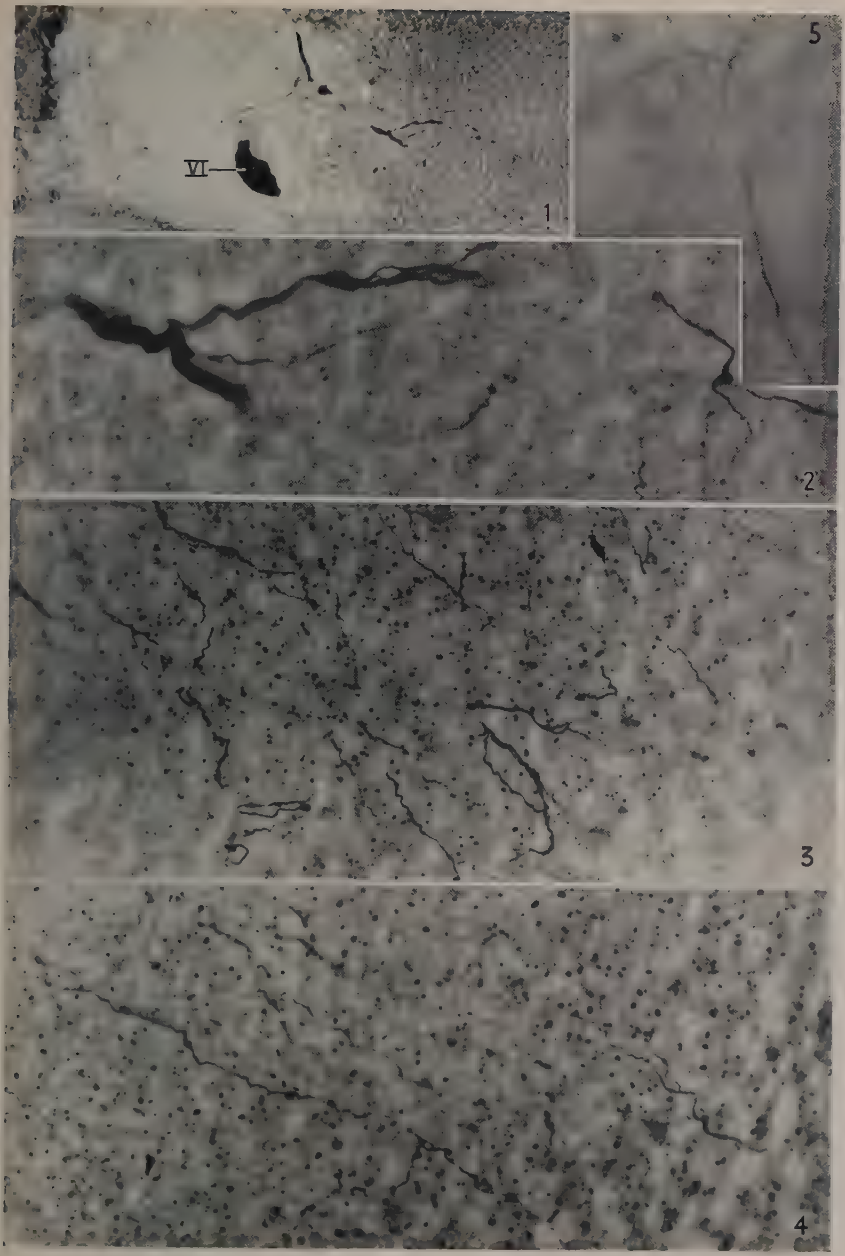
Fig. 12. Section of urogenital cord in a 46-mm. C.R. length male human foetus (H. 653). The fused and retrogressing paramesonephric ducts (*p*) appear as an epithelial cord in the centre of the microphotograph. On either side of this cord the well-formed mesonephric ducts (*m*) can be seen. ×108.

Fig. 13. Section of urogenital cord in a 46-mm. C.R. length female human foetus (H. 679). The fused paramesonephric ducts (*p*) constitute the central uterovaginal canal, on either side of which can be seen the mesonephric ducts (*m*). The epithelium of these ducts is separated from the underlying connective tissue and is showing early retrogressive changes. The mesodermal condensation round the ducts is much more extensive and denser than in the male specimen of the same developmental stage illustrated in fig. 12. ×108.

Fig. 14. Longitudinal section through *uterus masculinus* (*p*) in a 92-mm. C.R. length male human foetus (H. 125, de Castro technique). Note large nerve fibre in epithelium of *uterus masculinus* and absence of such fibres in the basa deferentia (*m*). ×108.

Plate 4

Fig. 15. Transverse section through urethra of an 84-mm. C.R. length male human foetus (H. 178, de Castro technique). Note bundle of nerve fibres passing through epithelium of *uterus masculinus* (*p*) to enter its lumen. Such fibres are absent from the common ejaculatory ducts (*m*), from the developing prostatic tubules, and from the epithelium of the urethra itself. ×81.



J. D. BOYD *and* A. F. W. HUGHES

Plate 1

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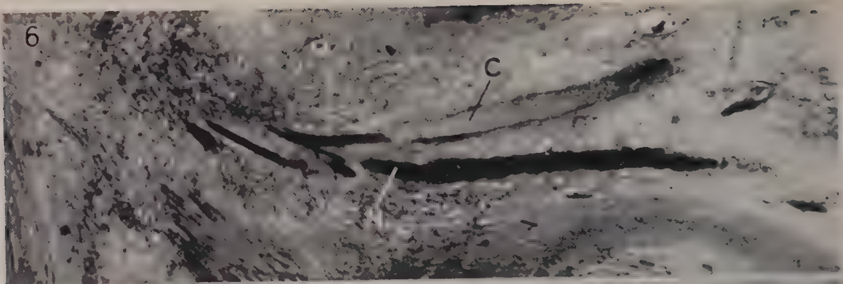

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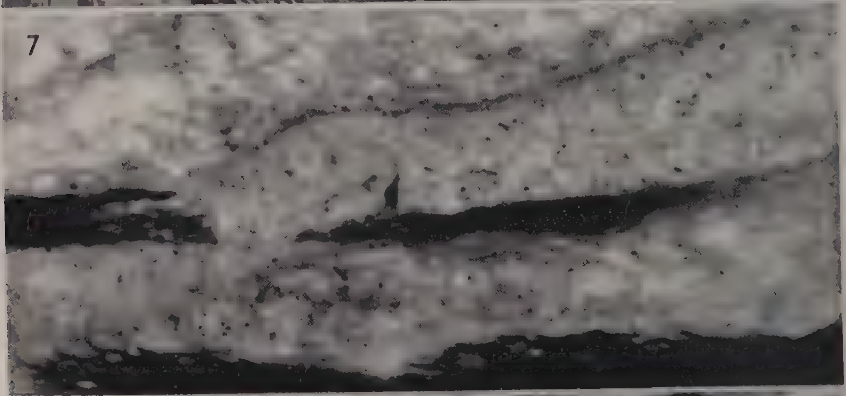

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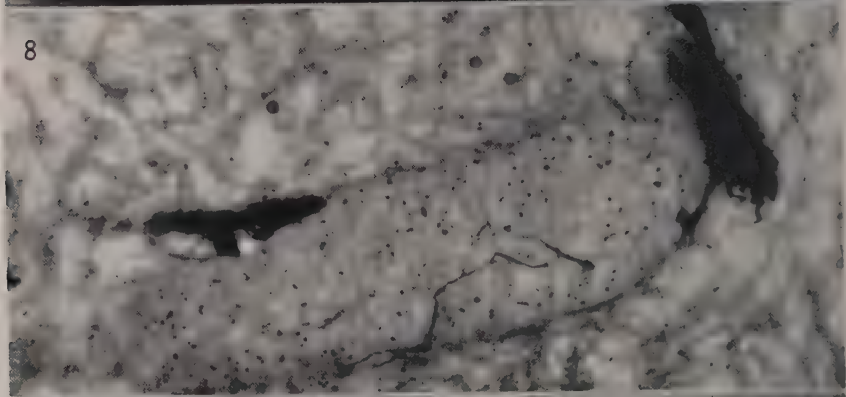

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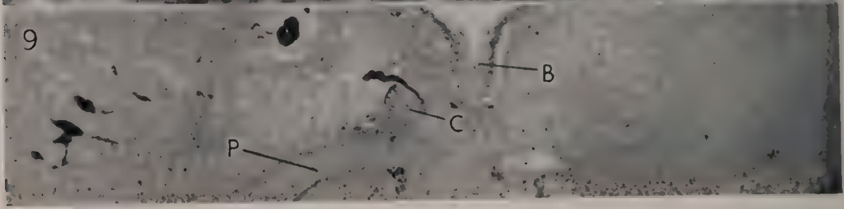

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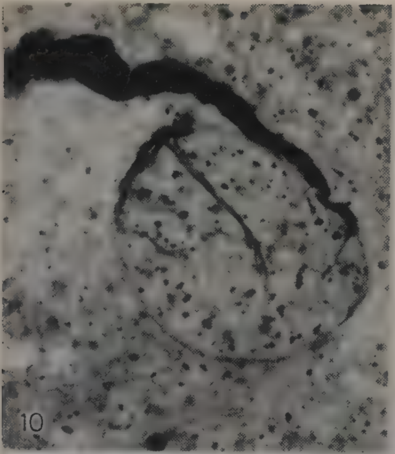
Plate 2



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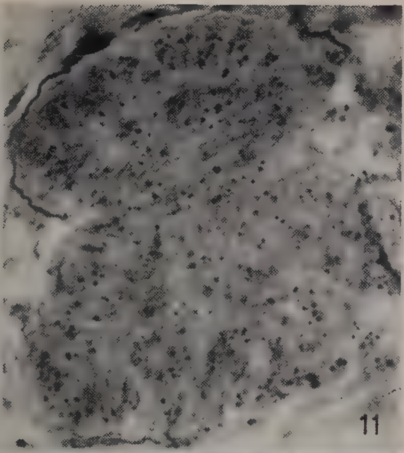

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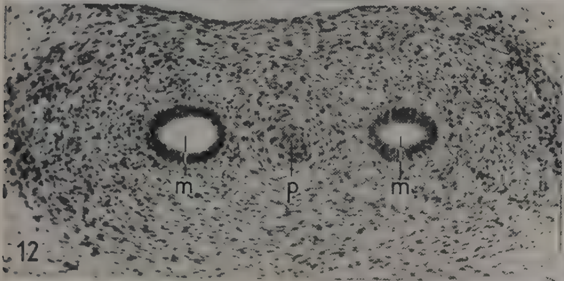

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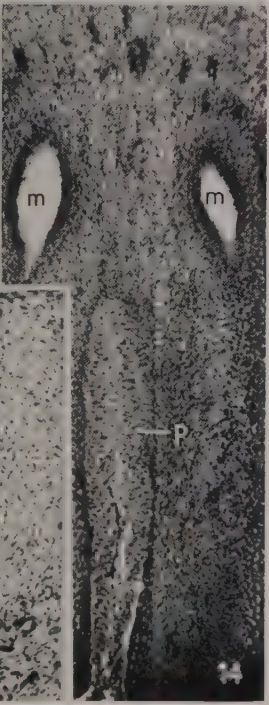

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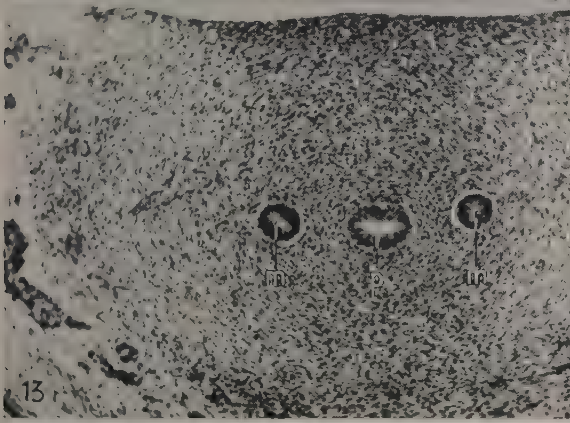

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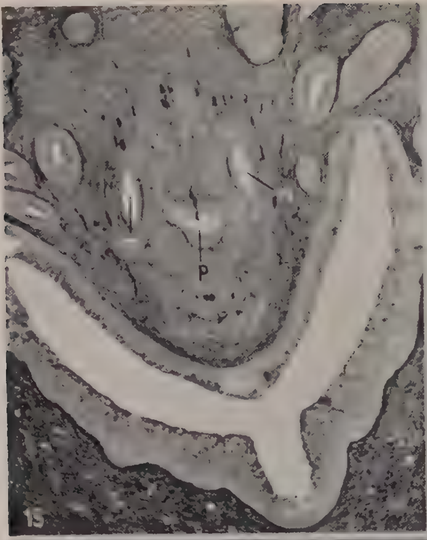
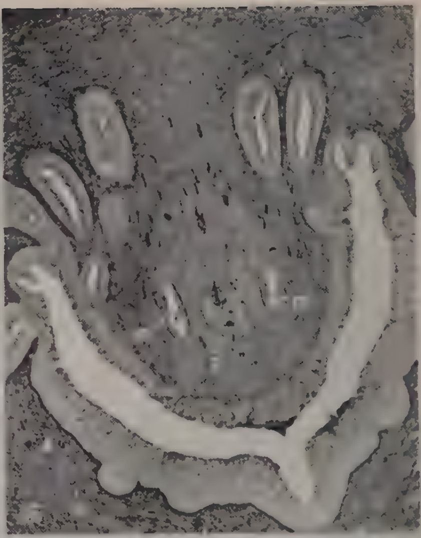
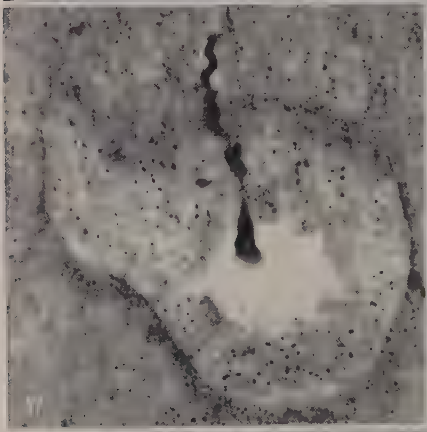
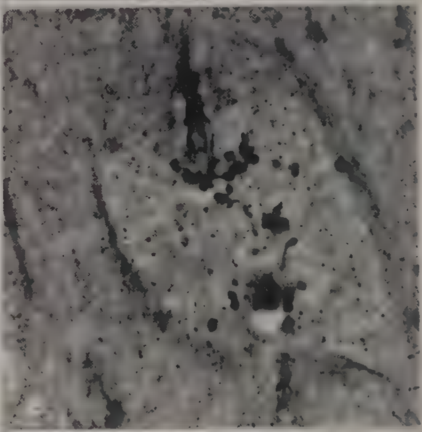
Plate 3









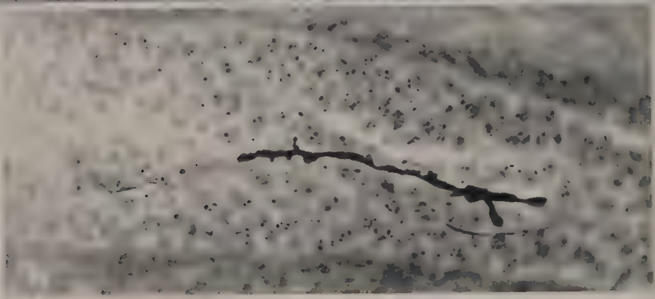


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Plate 4

FIG. 16. Transverse section through urethra of same foetus illustrated in fig. 15, but at a more caudal level. Here the *uterus masculinus* (*p*) is a solid epithelial rod with numerous nerve fibres. Such fibres are absent from adjacent epithelium, including that of the common ejaculatory ducts (*m*). ×81.

FIG. 17. High-power view of *uterus masculinus* in fig. 15. The bundle of nerve fibres can be seen passing into lumen of the vestigial organ. ×513.

FIG. 18. High-power view of *uterus masculinus* in fig. 16. Note the numerous end-bulbs. ×513.

FIG. 19. High-power view of *uterus masculinus* in fig. 14 to show the terminal ramification of a swollen nerve fibre amongst the epithelial cells. ×513.

(Manuscript received 24:ix:59)

Les Malformations cardiaques obtenues par l'irradiation aux rayons X du cœur de l'embryon de Poulet

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AVEC DEUX PLANCHES

DANS un travail antérieur (Le Douarin & Le Douarin, 1959), nous avons étudié les répercussions de l'irradiation du cœur de l'embryon de Poulet sur l'évolution des arcs aortiques. Par ailleurs, les rayons X exercent sur le cœur lui-même une action tératogène.

TECHNIQUE

Les œufs (Leghorn blanc) ont été ouverts au 3ème jour de l'incubation (Stades 16 à 18 de Hamburger et Hamilton) et l'irradiation localisée au cœur a été effectuée selon la technique mise au point par Wolff (1936). L'emploi d'un tube localiseur et d'un écran, ne laissant à découvert que le cœur de l'embryon, rend impossible la mesure précise de la dose de rayonnement reçue par l'organe. Mais le temps d'exposition est, toutes autres choses égales, proportionnel à cette dose et peut donc servir de critère valable si l'on utilise toujours le même appareil dans des conditions de fonctionnement constantes.

Ce travail a été effectué avec l'appareil américain Picker X ray, anti-cathode de tungstène de 4 mm.[2], sous une tension de 60 KV et à une intensité de 8 mA. Dans ces conditions, une irradiation de 6 minutes permet d'obtenir un pourcentage notable de survie au-delà du 13ème jour de l'incubation. Seuls les embryons ayant franchi ce seuil ont été étudiés pour ce travail, de telle sorte que les malformations décrites ne puissent être attribuées à un simple retard dans le développement. Soixante embryons ont pu être ainsi examinés, 46 étaient porteurs de malformations intra-cardiaques et d'anomalies de la morphologie externe.

MORPHOLOGIE EXTERNE

Les cœurs ayant été soumis à l'irradiation sont généralement d'une taille supérieure à la normale, cette hypertrophie étant d'ailleurs variable suivant les

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cas. Elle est parfois considérable comme le montre l'exemple de la figure 1 de la Planche 1 (on y voit que le cœur occupe la majeure partie de la cavité du tronc).

Du point de vue de la forme de l'organe, on peut classer les cœurs obtenus en 3 catégories: cœurs normaux, 14 cas sur 60; hypertrophie gauche, 40 cas sur 60; cœurs bifides, 6 cas sur 60.

Cœurs de forme normale. Ils peuvent cependant, dans certains cas, présenter des malformations internes.

Hypertrophie du cœur gauche. Elle porte sur le ventricule et sur l'oreillette. Cette dernière est souvent rabattue latéralement. Cette hypertrophie gauche s'accompagne d'une réduction, souvent très importante, du volume du ventricule droit (Pl. 1, fig. 2).

Cœurs bifides. Les deux ventricules sont alors développés, et leurs sommets sont séparés par une incisure.

Par ailleurs, le développement exagéré du cœur gauche peut s'accompagner d'une position anormale du bulbe et du tronc artériels, ceux-ci n'étant pas situés sur la ligne médiane, mais restant en avant de l'oreillette droite qui est alors peu développée (Pl. 1, fig. 3).

CONSTITUTION INTERNE

Son étude a été faite à l'aide de coupes transversales pratiquées à différents niveaux. Les figures 4 et 5 de la Planche 1 en représentent l'aspect normal, chez un témoin. La figure 4 montre l'aspect de la coupe passant par le canal atrio-ventriculaire droit et la rampe pulmonaire, la figure 5 celui de la coupe pratiquée au niveau des valvules semi-lunaires.

Anomalies du cloisonnement du tronc et du bulbe artériels

Plusieurs degrés peuvent s'observer dans la malformation de la cloison aortico-pulmonaire.

Bulbe indivis. Le bulbe est alors représenté par une cavité assez vaste, située au-dessus de la cloison inter-ventriculaire. Celle-ci est interrompue par le foramen inter-ventriculaire non obturé. Dans certains cas, la paroi bulbaire présente des ébauches de crêtes, mais elles sont réduites et il n'existe pas de valvules semi-lunaires. Cette disposition anatomique implique un mélange complet des sangs propulsés par les deux ventricules.

Ce bulbe indivis est généralement en situation médiane, mais il peut se trouver au-dessus du ventricule droit par suite d'une insuffisance de la rotation apparente qu'il effectue normalement. Il peut se prolonger par un tronc normalement cloisonné (Pl. 2, fig. 6), ou, au contraire, le défaut de formation de la cloison aortico-pulmonaire peut se continuer au niveau de la base du tronc artériel, le bulbe donnant alors accès à un canal unique qui se ramifie plus loin selon une disposition plus ou moins proche de la normale. La figure 7 de la Planche 2 montre un exemple de ce dernier cas; on voit qu'il n'existe pas de valvules semi-lunaires, mais seulement des bourrelets situés avant la ramification du tronc artériel.

Crêtes bulbaires non soudées. Les crêtes bulbaires A et B peuvent subir un début de développement sans arriver à se rencontrer et à se souder sur la ligne médiane. Il en résulte encore une possibilité de mélange des sangs aortique et pulmonaire qui ne sont pas séparés par une cloison anatomique continue.

Foramen au niveau de la cloison bulbaire. Dans ce cas, le bulbe a une constitution normale et en particulier possède des valvules semi-lunaires, mais juste au-dessous de celles-ci un orifice plus ou moins important traduit une soudure imparfaite des crêtes de la portion proximale du bulbe. Il est évident que le mélange sanguin est ici aussi possible, mais moins facilement que précédemment.

Malformation de la rampe aortique: foramen inter-ventriculaire persistant

Normalement, le foramen inter-ventriculaire est utilisé pour connecter le tronc aortique et le ventricule gauche. Ceci est rendu possible par le fait que le bulbe devient ventral par rapport au canal atrio-ventriculaire, et à droite de celui-ci. L'orifice bulbo-ventriculaire se trouve donc au-dessus et à droite du foramen inter-ventriculaire, et le bulbe communique ainsi avec les deux ventricules. Le sang du ventricule gauche est chassé à chaque systole dans le tronc aortique, à travers l'orifice inter-ventriculaire, tout le long d'une rainure creusée du côté droit du coussinet endocardique du septum inter-auriculaire. Cette rainure communique donc initialement avec le ventricule droit, mais lors de l'achèvement de la rampe aortique elle est transformée en un canal par une expansion du matériel endocardique. Ainsi, les deux rampes artérielles cessent de communiquer entre elles.

La malformation de la rampe aortique par persistance de l'orifice inter-ventriculaire consiste donc en une non-transformation en canal de la gouttière du coussinet endocardique médian. La figure 8 de la Planche 2 montre un exemple de cette anomalie fréquente. On voit que le ventricule droit peut lui aussi alimenter la rampe aortique.

Dispositions bulbaires anormales

Les croissances différentielles des parties du tube cardiaque initial se traduisent, au niveau du bulbe, par deux mouvements apparents. D'une part, le bulbe situé initialement à droite gagne la ligne médiane et le cœur retrouve une apparence de symétrie. D'autre part, la cloison bulbaire qui se forme est spiralée, de telle sorte que la rampe pulmonaire issue du ventricule droit passe graduellement à gauche de la rampe aortique.

Ces deux aspects de l'évolution du bulbe peuvent être perturbés.

Déplacement inachevé du bulbe vers la ligne médio-ventrale. La figure 3 de la Planche 2 en montre un exemple. Lorsque cette anomalie se rencontre, l'oreillette droite, qui est en situation directement dorsale par rapport au bulbe, est réduite. Ceci semble constituer un argument en faveur de la théorie selon laquelle le bulbe gênerait la croissance de cette oreillette, au début du développement.

Cette position anormale du bulbe coexiste souvent avec d'autres malformations de la constitution interne. C'est ainsi que l'exemple cité est celui d'un embryon qui présentait par ailleurs un orifice inter-ventriculaire non obturé, une rampe aortique réduite à une gouttière confluant avec la rampe pulmonaire pour former avec cette dernière un bulbe artériel indivis.

Anomalie de la spiralisation de la cloison. La spiralisation des rampes artérielles peut être exagérée, la rampe aortique se trouvant alors ventralement et à droite de la rampe pulmonaire.

Cette disposition, associée à d'autres malformations internes, peut créer des conditions circulatoires très aberrantes, en particulier lorsque le bulbe est indivis ou imparfaitement cloisonné. Dans ces conditions en effet, le ventricule gauche peut facilement alimenter les deux rampes, tandis que le sang du ventricule droit passe plus aisément dans la rampe aortique que dans la rampe pulmonaire.

Réduction du ventricule droit

La réduction du ventricule droit qui accompagne l'hypertrophie du cœur gauche peut atteindre des proportions très importantes.

Les arcs 6 reçoivent alors du sang du ventricule gauche par suite d'une malformation bulbaire permettant un échange sanguin.

Par exemple, la figure 7 de la Planche 2 montre le tronc indivis d'un embryon dont le ventricule droit, extrêmement réduit, ne présentait qu'une rampe pulmonaire très petite confluant avec la rampe aortique.

Cependant, un certain degré de réduction du ventricule droit peut exister seul, sans qu'il y ait de communication aortico-pulmonaire.

Anomalies des valvules auriculo-ventriculaires

On sait que l'orifice atrio-ventriculaire gauche possède une valvule septale et deux valvules marginales. L'orifice droit possède une valvule marginale qui est envahie par du muscle et constitue un grand repli de la paroi ayant la forme d'un croissant dans la concavité duquel se trouve l'orifice atrio-ventriculaire. La valvule septale est ici réduite à un simple bourrelet. La figure 4 de la Planche 1 montre, chez un témoin, la disposition du canal atrio-ventriculaire droit et sa valvule marginale.

Les anomalies valvulaires observées consistent en une réduction de leur importance, la valvule marginale droite en particulier pouvant être de hauteur réduite ou même n'être qu'un simple bourrelet. Dans les cas extrêmes, la suppression des valvules est totale.

Anomalies des parois (Étude macroscopique)

Parois ventriculaires. Les cavités des ventricules peuvent être vastes, les parois étant plus minces que normalement et leur face interne lisse. Dans ces conditions, l'hypertrophie cardiaque n'est pas due à une hyperplasie musculaire, mais peut au contraire s'interpréter comme étant une distension par la pression sanguine, des parois ventriculaires physiologiquement insuffisantes. La figure 9 de la Planche 2 montre un tel cas de cavités ventriculaires vastes et à parois minces.

Parois auriculaires. On observe dans de nombreux cas et la plupart du temps au niveau de l'oreillette gauche, des parois épaisses et très fortement plissées.

Malformations associées

Les différentes malformations décrites peuvent coexister chez un même embryon, et leur groupement aboutir à la réalisation de cœurs monstrueux, ne répondant même plus au plan d'organisation normal de celui des Oiseaux, comme l'exemple suivant va le montrer: il s'agit d'un embryon dont le cœur présentait au point de vue de la morphologie externe une saillie du ventricule droit et un bulbe artériel en relief. Les figures 9 et 10 de la Planche 2 représentent deux coupes transversales de ce cœur passant par le canal atrio-ventriculaire droit (fig. 9: vue vers les ventricules; fig. 10: vue vers les oreillettes). On voit que les deux cavités ventriculaires sont grandes, à parois minces et lisses. Le ventricule droit ne présente pas en coupe transversale la forme normale d'un croissant recouvrant ventralement le ventricule gauche.

L'atrium n'a pas complètement effectué le mouvement de bascule qui l'amène normalement en position crâniale par rapport aux ventricules, mais une partie de l'oreillette droite se trouve en arrière du ventricule si bien que l'orifice atrio-ventriculaire droit n'est pas dans un plan horizontal, mais très oblique.

La valvule atrio-ventriculaire droite est réduite à un bourrelet; l'orifice atrio-ventriculaire gauche ne possède qu'une valvule septale réduite. Or, on explique le modelage des valvules à partir des bourrelets endocardiques primitifs, par la pression sanguine des systoles. Dans le cœur considéré cette pression devait être faible, étant donnée l'hypoplasie musculaire. On peut donc invoquer cette raison dans l'inachèvement valvulaire observé.

Par ailleurs, l'incorporation du bulbe artériel aux ventricules est incomplète: le bulbe demeure volumineux et saillant, sa paroi est mince et sa cavité vaste. Il est indivis dans sa partie proximale, les crêtes A et B sont ébauchées mais peu développées. Elles contribuent cependant à former au niveau du bulbe deux gouttières superposées; l'une, dorsale, allant du ventricule gauche au tronc aortique, et l'autre, ventrale, allant du ventricule droit au tronc pulmonaire. Donc, le sang du bulbe avait tendance à former deux courants superposés mais non séparés par une cloison anatomique. Enfin, ce bulbe se trouve placé au-dessus du foramen inter-ventriculaire largement ouvert; il communique également avec l'oreillette droite comme on peut le voir sur la fig. 10.

Rapports entre les anomalies vasculaires et les malformations cardiaques

En considérant les malformations internes du cœur d'une part, et les anomalies des arcs aortiques d'autre part, les quatre combinaisons possibles ont été rencontrées:

cœur normal et arcs normaux	9 cas (irradiation inefficace);
cœur normal et arcs anormaux	5 cas;
cœur anormal et arcs normaux	12 cas; et
cœur anormal et arcs anormaux	34 cas.

Arcs anormaux et cœurs normaux. Dans la totalité des cas, les anomalies vasculaires n'impliquent pas de modification de la circulation intra-cardiaque, puisqu'il s'agit:

de persistance de l'arc 4 gauche et de la racine aortique droite: 1 cas; d'anomalies du tronc brachiocéphalique droit: 3 cas; et d'arc 6 droit de diamètre réduit: 1 cas.

Cœurs anormaux et arcs normaux. On peut rencontrer dans cette catégorie les différentes anomalies cardiaques décrites, qui peuvent donc exister en dehors de toute malformation vasculaire.

Cœurs et arcs anormaux. Wilson & Warkany (1949) ont montré sur l'embryon de rat que la cloison aortico-pulmonaire est incomplète lorsque la crosse aortique ou la crosse pulmonaire fait défaut. Stéphan (1952) confirme ces résultats sur l'embryon de Poulet.

Chez les cœurs irradiés, lorsque la crosse aortique est absente, la nécessité d'une communication aortico-pulmonaire est d'autant plus impérative que le ventricule droit est plus réduit. En effet, la plus grande partie du sang de la circulation de retour arrive dans l'oreillette droite et de là, passe dans les deux ventricules (la cloison inter-auriculaire étant perforée). Lorsque l'aorte dorsale est formée par la confluence des arcs 6, il devient donc nécessaire que le ventricule gauche puisse chasser une partie notable de son sang dans le tronc pulmonaire, et ceci d'autant plus que le ventricule droit est réduit.

Cependant, l'absence de crosse aortique sans communication aortico-pulmonaire a été rencontrée une fois, mais le cas est très différent: il s'agit d'un embryon qui était couché sur le côté droit lors de l'irradiation. On observe une réduction du ventricule gauche tandis que le droit est très développé. Les arcs aortiques sont anormaux:

à droite, l'arc 4 donne la carotide et le 3 la sous-clavière; et à gauche, l'arc 3 est absent et le tronc brachiocéphalique est fourni par l'arc 6.

Dans ces conditions, le ventricule gauche ne fournit de sang qu'aux territoires irrigués par la carotide et la sous-clavière droites, le reste de la circulation générale ainsi que la circulation pulmonaire étant assurés par le ventricule droit.

CONCLUSION

1. L'irradiation du cœur de l'embryon de Poulet de 3 jours entraîne généralement une hypertrophie cardiaque, qui siège le plus souvent à gauche. Au niveau

du ventricule, l'hypertrophie peut tenir à un grand développement du muscle, ou bien, c'est la cavité qui est anormalement grande, la paroi étant mince. L'oreillette gauche est très volumineuse chez la plupart des cœurs irradiés.

Donc, de même que les arcs droits sont plus fréquemment perturbés que les gauches, le cœur droit est plus atteint par l'irradiation. Ceci peut être dû à la position habituelle de l'embryon, couché sur le côté gauche lors de l'intervention, et explique peut-être le grand développement du cœur gauche qui, moins atteint, pourrait présenter une hypertrophie compensatrice.

2. Les malformations internes du cœur (obtenues dans 46 cas sur 60) sont les suivantes: (*a*) Cloisonnement incomplet du bulbe et du tronc artériels (Pl. 2, figs. 6 et 7); (*b*) Persistance du foramen inter-ventriculaire (Pl. 2, fig. 8); (*c*) Rotation et torsion anormales du bulbe artériel (Pl. 1, fig. 3); (*d*) Inachèvement des valvules à partir du matériel endocardique (Pl. 2, fig. 9).

Les phénomènes morphogénétiques au niveau du cœur s'échelonnant du $3^{\text{ème}}$ au $8^{\text{ème}}$ jour de l'incubation, l'irradiation peut donc avoir des effets lointains.

Les perturbations de l'évolution du matériel endocardique, très important dans le cloisonnement des cavités cardiaques, indiquent une sensibilité particulière de ce tissu.

3. Les malformations des arcs aortiques peuvent exister en dehors de toute anomalie cardiaque, mais dans certains cas leur association réalise des conditions circulatoires extrêmement aberrantes.

4. Du point de vue physiologique, une grande partie des malformations internes observées provoquent des mélanges sanguins plus ou moins importants. Dans certains cas les dispositions anatomiques sont telles que les courants sanguins créés par les systoles se mélangent obligatoirement. Il faut noter que des malformations aussi considérables restent compatibles avec une survie prolongée de l'embryon et même éventuellement avec une éclosion.

SUMMARY

In the chick embryo, internal malformations of the heart and anomalies in its external morphology are caused by X-irradiation of this organ.

The irradiation was accomplished on the third day of incubation using an American Picker X-ray machine (anticathode 4 mm.^2): the operating potential was 60 kV, the current was 8 mA, and the exposure lasted for 6 minutes. The only embryos examined were those surviving to at least the 13th day of incubation.

1. Irradiation generally causes hypertrophy of the heart, usually localized to the left side (Pl. 1, figs. 1, 2). At the ventricular level, hypertrophy is evidenced either by muscle enlargement or by the enlargement of the ventricular cavity accompanied by thinning of its wall (Pl. 2, figs. 9, 10).

In most cases, the left atrium is distended while the right side of the heart, especially the ventricle, becomes smaller. Thus, the right side of the heart is

more susceptible to X-irradiation than the left. Previous research has shown that the same is true of the aortic arches. The embryo is lying on its left side during irradiation, and consequently the left side of the heart is less affected by the treatment and shows a compensatory hypertrophy.

2. Malformations within the heart (which occurred in 46 out of 60 cases) may be categorized as follows:

(*a*) Incomplete partitioning of the bulbus and the arterial truncus (Pl. 2, figs. 6, 7, 10).

(*b*) Persistence of the interventricular foramen (Pl. 2, fig. 8).

(*c*) Anomalous rotation and twisting of the arterial bulbus (Pl. 1, fig. 3).

(*d*) Incomplete formation of the valves from endocardial tissue, the semilunar valves are very often absent or incomplete (Pl. 2, figs. 9, 10).

The aberrations in the development of the endocardial tissue (and hence of the aortico-pulmonary partitioning) show it to be especially sensitive to irradiation.

3. Malformations of aortic arches may exist apart from any anomaly of the heart itself, but in certain other cases the existence of dual malformations creates extremely aberrant circulatory conditions.

4. From a physiological standpoint, a great many of the internal malformations observed result in a more or less complete blending of blood-streams. In some cases, the anatomical dispositions are such that, following systole, the blood-streams are blended, chiefly at the bulbus level.

Such gross malformations are compatible with prolonged survival of the embryo, which can, occasionally, hatch.

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EXPLICATION DES PLANCHES

PLANCHE 1

FIG. 1. Vue ventrale d'un cœur très hypertrophié, occupant la plus grande partie de la cavité du tronc.

FIG. 2. Vue ventrale d'un cœur présentant une hypertrophie gauche et une réduction du ventricule droit. (On voit par ailleurs que les arcs aortiques sont anormaux: absence de crosse aortique.)

FIG. 3. Vue ventrale d'un cœur montrant une hypertrophie gauche et une situation anormale du bulbe, situé à droite et masquant l'oreillette.

FIG. 4. Témoin. Coupe transversale, vue vers les oreillettes. Niveau du canal atrio-ventriculaire droit et de la rampe pulmonaire. *VD, VG*: ventricules droit et gauche; *CD*: canal atrio-ventriculaire droit; *RP*: rampe pulmonaire; *vm*: valvule marginale.

FIG. 5. Témoin. Niveau des valvules semi-lunaires, vue vers les ventricules. *CD*, *CG*: canaux atrio-ventriculaires droit et gauche; *OD*: oreillette droite; *vA*, *vP*: valvules semi-lunaires aortiques et pulmonaires.

PLANCHE 2

FIG. 6. Coupe transversale d'un cœur irradié. Vue vers les oreillettes. *B*—bulbe indivis se prolongeant par un tronc cloisonné dont on voit le départ de la partie aortique (*A*) et de la partie pulmonaire (*P*).

FIG. 7. Coupe transversale d'un cœur irradié, au niveau des canaux atrio-ventriculaires droit et gauche. On voit le tronc artériel (*Ti*) indivis sur une certaine longueur et présentant des bourrelets sur sa face interne.

FIG. 8. Coupe transversale d'un cœur irradié. Vue vers les ventricules. Persistance du foramen inter-ventriculaire (*F*), faisant communiquer le ventricule droit et la rampe aortique (*RA*). On voit par ailleurs que le tronc pulmonaire (*P*) possède des valvules semi-lunaires réduites.

FIG. 9. Coupe transversale d'un cœur irradié. Vue vers les ventricules. Les ventricules droit et gauche (*VD* et *VG*) sont dilatés et communiquent par un large foramen inter-ventriculaire (*F*). Le ventricule droit n'a pas la forme normale en croissant. La valvule septale gauche (*vsg*) est réduite. Une partie importante de l'oreillette droite est restée en arrière du ventricule.

FIG. 10. Coupe transversale du cœur précédent, au même niveau. Vue vers les oreillettes. On voit le plafond du ventricule droit (*VD*), et en arrière de lui l'oreillette droite (*OD*). Le bulbe (*B*) est indivis. Le tronc aortique est cloisonné, on voit le départ de la partie aortique (*A*) et de la partie pulmonaire (*P*).

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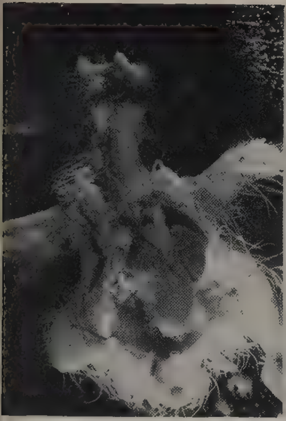

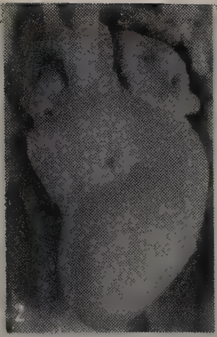

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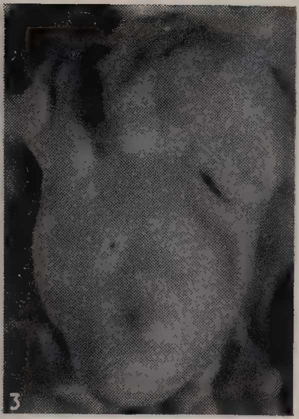

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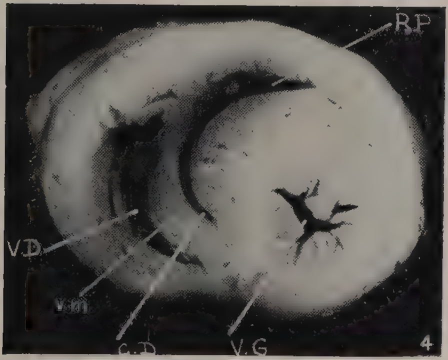

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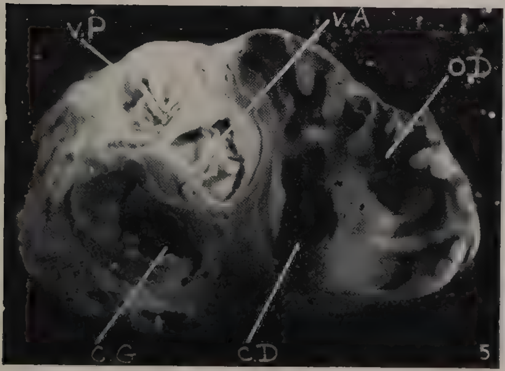

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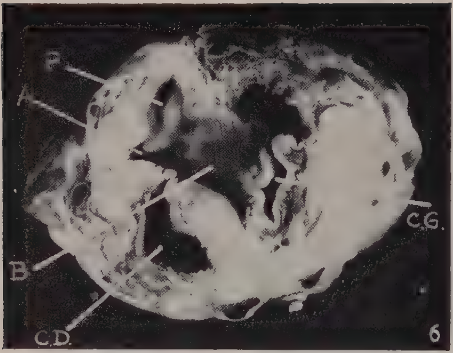
G. LE DOUARIN

Planche 1



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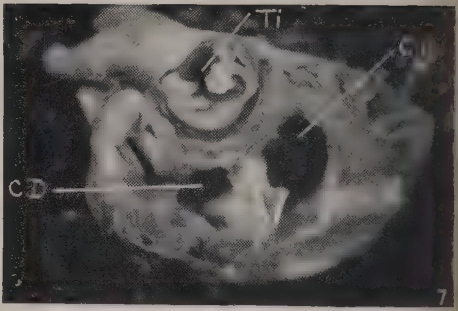

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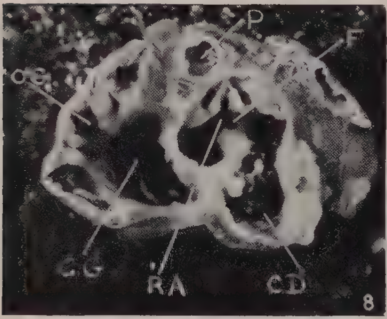

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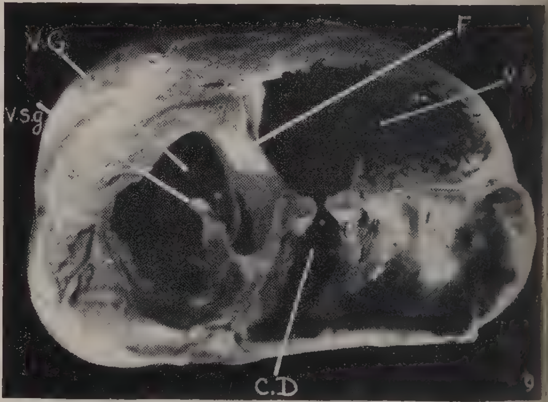

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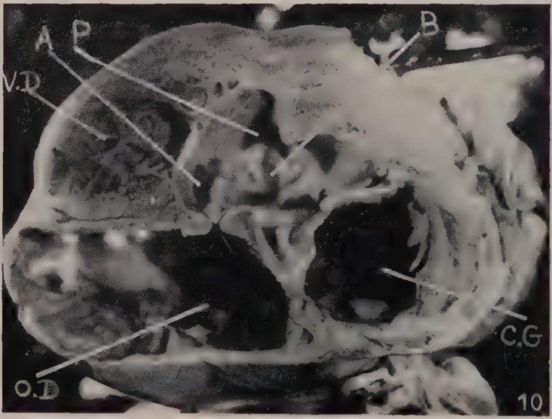

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G. LE DOUARIN

Planche 2

The Growth of the Locust Embryo

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From the Department of Zoology, University of Edinburgh

INTRODUCTION

It would appear that the growth of an insect embryo has never been measured, and, indeed, with the exception of Ranzi's (1929, 1930) work on *Sepia*, all the available information on embryonic growth pertains to vertebrates. The object of the present study was to obtain measures of the growth of the locust embryo. Such data, together with other relevant physical data on changes in the developing egg, are needed to provide a basis for further studies on the energetics of growth and development. It is also important to know whether the principles of vertebrate embryonic growth may be usefully applied to the insect embryo.

MATERIAL

The eggs used were those of *Locusta migratoria migratorioides* (R. & F.). They are laid in pods, 22–81 eggs per pod according to Roonwal (1936*a*), are among the largest of insect eggs (6–8 mm. length), and are highly telolecithal. The egg-pods were kept on moist cotton wool at 30° ±0·5° C. and they took 12 days to develop, the hoppers appearing on the thirteenth day. In Table 1 the appearances of the eggs and embryos at the end of each day of development are listed. Throughout this work the appearance of the eggs was noted in relation to their age. In any one pod eggs tended to be at the same stage (as defined in Table 1), with a few either more advanced or, more commonly, slightly retarded, in development. The numbers of instances at which each stage occurred on each day were recorded for all the pods. The occurrence of these different stages at each day of incubation is shown in Table 2 where each entry represents the occurrence in an egg-pod of a particular stage at a particular age. The distribution in Table 2 shows clearly the occurrence of a modal stage at each age (the sixth and eighth days are bimodal in this respect), and this has been used in assigning ages to the stages listed in Table 1. Recently Shulov & Pener (1959) have made a special study of the appearance and age of embryos of this species. At the end of Table 1 there has been included, therefore, a list of their stages which correspond with the embryonic stages and ages of the present work. They incubated their eggs at

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27° C. and at this temperature development extended over 16 days. The equivalencies of the two developmental stage schemata are satisfactory in view of the difference in incubation times. Shulov & Pener report greater ranges in the stages which occurred at each age than those given here. This may be due to the effects of the longer developmental time and to the more specific and restricted nature of the classes distinguished by these workers. Moreover, the egg-pods in the present work were usually collected every 6 hours as opposed to the morning collection practised by Shulov & Pener.

TABLE 1

Developmental stage and age of eggs of Locusta migratoria migratorioides *(R. & F.)*

Stage	*Appearance of egg and embryo*	*Days at 30° C.*	*Stages of Shulov & Pener (1959)*
1	Egg flaccid. Embryo cap of cells at posterior pole	1	I–II
2	Egg flaccid. Embryo small; protocephalon protocorm, segments appearing	2	III–V
3	Egg flaccid. Embryo long, thin; segmentation complete; appendages forming	3	VI–VIII
4	Egg turgid. Embryo fatter; appendages segmented	4	IX–XII
5	Egg fully turgid. Embryo large, abdomen slightly twisted, moving away from posterior pole of egg; uric acid crystals appearing in abdomen	5	XIII–XV
$5\frac{1}{2}$	Katatrepsis in process, space at micropylar end of egg; embryo bent around yolk, appendages lengthening	6	XVI–XVII
6	Egg slightly pear-shaped. Embryo facing anterior pole of egg, appendages lengthening	6	XVII–XVIII
7	Embryo $\frac{1}{2}$ to $\frac{2}{3}$ length of egg	7	XVIII
$7\frac{1}{2}$	Embryo $\frac{3}{4}$ length of egg	8	XIX
8	Embryo full length of egg, yolk totally enclosed; hind femora cover 3–4 abdominal segments	8	XX
9	No pigment; hind femora cover 5 abdominal segments	9	XXI
10	Pigment appearing; hind femora full length covering 6 abdominal segments	10	XXI–XXII
11	Heavy pigment; pigmentation appearing on spines of hind tibiae	11	XXII
12	Spines on tibiae heavily pigmented	12	XXIII
13	Hopper stage	13	—

Measurements of the whole egg were made from the first day of development, and of the embryos from the end of the third day of incubation because embryos younger than this were too small and fragile to handle. Third- and fourth-day embryos were dissected from the egg in 0·5 per cent. NaCl solution in order to prevent the swelling and disintegration that occurred in distilled water. Embryos in stages 3–$7\frac{1}{2}$ (Table 1) were washed free of yolk. In stages 8–12 the yolk had become enclosed by the embryo and had to be squeezed out through a small slit made in the dorsal surface of the embryo. When possible, a series of measurements was made on the eggs from a single pod. In this way six eggs could be measured each day. During the first 3 days of development it was not possible to dissect out all the eggs from a pod because they were easily damaged. Each pod, therefore, was broken into four portions and eggs taken from the exposed surfaces, so that each sample contained smaller eggs from the ends of the pod as well as the larger centrally placed eggs. On the fourth day all the eggs were dissected out from the pod and subsequent samples taken at random. The few eggs that developed faster or slower, as judged by their appearance, than the rest of the pod (Table 1), were rejected. In one case (dry weight of egg) mixed samples from several pods had to be used, and in another case (reduced weight of egg) the same eggs were measured throughout their development. The latter instance was the only technique in which there was very little possibility of damage or shock to the developing egg.

TABLE 2

Relation between age and appearance of eggs. Entries show the number of pods whose eggs presented any particular stage at any particular age

Number of stage	*Age of egg-pods in days of incubation at 30° C.*															*Age equivalent of stages*
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	
0																0
1		1														1
2		5	20													2
3			8	30	3											3
4					21	1										4
5						24	7									5
5½						3	16									6
6						1	23	5								6
7							10	21	1							7
7½								16	9	1						8
8								5	26	3						8
9									2	17						9
10									1	9	19					10
11											8	22				11
12												7	19			12
13													6	18	2	13

METHODS

Five parameters were measured.

1. *Wet weight*

(*a*) *Eggs.* Each egg was washed, dried with filter paper until all the surface moisture had just disappeared, and then quickly weighed to the nearest 0·1 mg. on a 50-mg. torsion balance at 20° C.

(*b*) *Embryos.* The wet weight of embryos was not measured directly but was estimated from the other measurements (see Discussion).

2. *Dry weight*

(*a*) *Eggs.* Eggs were dried in an air oven at 105° C. for 4 hours and quickly weighed one at a time on a 5-mg. torsion balance (correct to ±0·01 mg.) at 20° C. After a further 4 hours of drying they were again weighed. The mean of the two weighings was then taken. Before drying, the wet weight of the eggs was determined so that the water content could be estimated.

(*b*) *Embryos.* Batches of embryos from six pods were used in each of two series of measurements. The number of embryos in each batch varied from 50 on the third and fourth days to single embryos by the tenth or eleventh day. Each batch was dried at 105° C. in a weighed tinfoil cup for two periods of 3 hours. Weighings were made with 5- and 50-mg. torsion balances as before and the mean of the two weighings taken.

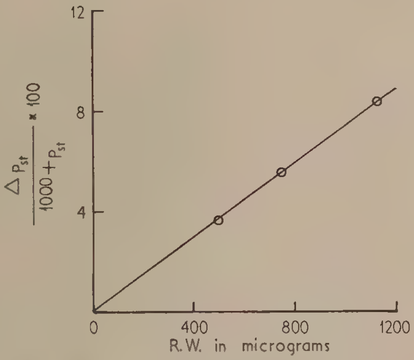
3. *Reduced weight*

Reduced weight or R.W. is the weight of an object in water, and this was determined by means of a Zeuthen Cartesian Diver Balance, similar to that described by Zeuthen (1948).

The only novel features of this apparatus were the use of polythene to form the cup on the glass diver and the use of small hollow glass beads as standard weights. The R.W. of these standards had been calculated from their density (determined in the density gradient described below) and their weight in air. These standards were accurate to ±1·0 microgram (μg.). One novel feature of the weighing procedure requires description. The formula for the diver balance given by Løvtrup (1950*a*) is

$$\text{R.W.}_{x} = Z \, . \, \frac{-\Delta p_x}{1000+p_x},$$

where R.W.$_{x}$ is the reduced weight of the unknown, Z is the volume of the air-bubble of the unloaded diver at equilibrium position, p_x is the difference in pressure required to restore equilibrium of the loaded diver, and $1000+p_x$ represents the new equilibrium pressure in Brodies. This formula is an equation for a straight line passing through the origin with slope Z, i.e. $Y = mX$, where $Y = \text{R.W.}_{x}$, $X = \frac{-\Delta p_x}{1000+p_x}$, and $m = Z$. This fact was employed in the weighing procedure. In making a set of weighings a series of standards was weighed and the values of R.W.$_{st}$ plotted against $\frac{-\Delta p_{st}}{1000+p_{st}}$ to give a straight line calibration graph for the diver. Text-fig. 1 is a typical calibration graph. Embryos or eggs were then measured and their R.W. read off the graph according to their values for $\frac{-\Delta p_x}{1000+p_x}$. Usually the unloaded diver pressure p_D remained constant and was checked after every three or six weighings. (If p_D should alter through the presence of debris on the diver or for any other reason, then provided the new value of p_D is measured it enters into the expression $\frac{-\Delta p_x}{1000+p_x}$ since this is $\frac{-(p_r-p_D)}{1000+p_D+\Delta p_x}$, while the change in Z, the volume of the diver bubble at equilibrium, is negligible for very small changes in p_D. Large alterations in Z occurred over several days and so did not affect any one set of weighings.) The standard weights were measured again at the end of each set of weighings to give further confidence in the calibration graph and to see whether there had been any change in Z, i.e. in the slope of the graph. Very rarely indeed was it necessary to repeat a set of weighings.



TEXT-FIG. 1. A typical calibration curve for the Cartesian Diver Balance. The points refer to the glass standard weights; p_{st} is the equilibrium pressure in centimetres of Brodie for the loaded diver and Δp_{st} is the difference between this value and that for the unloaded diver.

(*a*) *Eggs.* A special large diver was required for these measurements and its sluggish response reduced the sensitivity to ±10 μg. In each series twelve eggs were selected from a single pod and weighed every day. Any that failed to develop normally were discarded.

(*b*) *Embryos.* These were weighed singly from the third day onwards.

4. *Density*

Density measurements were made with a density gradient method similar to that described by Løvtrup (1950*b*), except that thorotrast-distilled water or thorotrast-saline gradients were used at 20° ±0·01° C. and calibrated with small (1–2 mm.) hollow glass beads previously made and measured to ±0·0005 g./ml. against droplets of standard KCl solutions in bromobenzene-kerosene gradients. The thorotrast gradients were about 10 cm. in height and covered the density ranges of 1·04–1·08 and 1·09–1·12 g./ml. (Thorotrast is a stabilized colloidal solution of thorium oxide, has a high density, low viscosity, negligible osmotic pressure, and is relatively transparent. It has been used for gradients in the Carlsberg Laboratory.)

(*a*) *Eggs.* Different eggs were used each day in thorotrast-distilled water gradients since development appeared to be affected by the treatment especially during the first three days.

(*b*) *Embryos.* Young embryos deteriorated rapidly in distilled water and so the gradients were made saline (0·75 % NaCl). In spite of this, third- and fourth-day embryos tended to continue falling in the gradient suggesting that they were taking up the thorotrast medium. In these cases an end-point was obtained by extrapolating back from the final slow steady rate of fall.

5. *Nuclear number*

(*a*) *Eggs.* No attempt was made to use the whole egg in these measurements because of the maceration difficulties presented by the chorion and the difficulty of obtaining suitable nuclear suspensions with the mass of yolk present.

(*b*) *Embryos.* Nuclei of single embryos were isolated in known volumes of macerating fluid and the total number of nuclei present estimated from haemocytometer counts of samples. The embryos were macerated in citric acid ($\frac{1}{3}$ M) with thymol and Shirlan N.A. (a proprietary fungicide made by I.C.I.) added to prevent bacterial and fungal growth. Each embryo was taken up in a graduated braking pipette with the macerating fluid and placed in a small glass bottle (7 or 10 ml.). The volume of the macerating fluid was then adjusted to give a nuclear concentration suitable for counting in a white blood-cell counting chamber. A known volume of an acid-fast nuclear stain, either Celestin Blue or Gallocyanin, was added and the bottles closed with waxed corks. After 24 hours the bottles were shaken at 50 c./s. for 30 minutes. Before sampling the bottles were vibrated for $\frac{1}{2}$–1 minute, and then slowly revolved to allow the air-bubbles to disperse. Sample counts were of the order of 250–500 and three samples were counted for each embryo, giving a total count of the order of 750–1,000. A count of this order should give an error of less than 10 per cent. The error is increased by incomplete maceration and small clumps of 2–5 nuclei did occur, especially in the case of older embryos. These older embryos were teased in their bottles before vibration.

RESULTS

All the results are presented as graphs of mean values. The full data are contained in a Ph.D. thesis on 'The Growth of the Insect Embryo', 1955, which is deposited in the library of the University of Edinburgh.

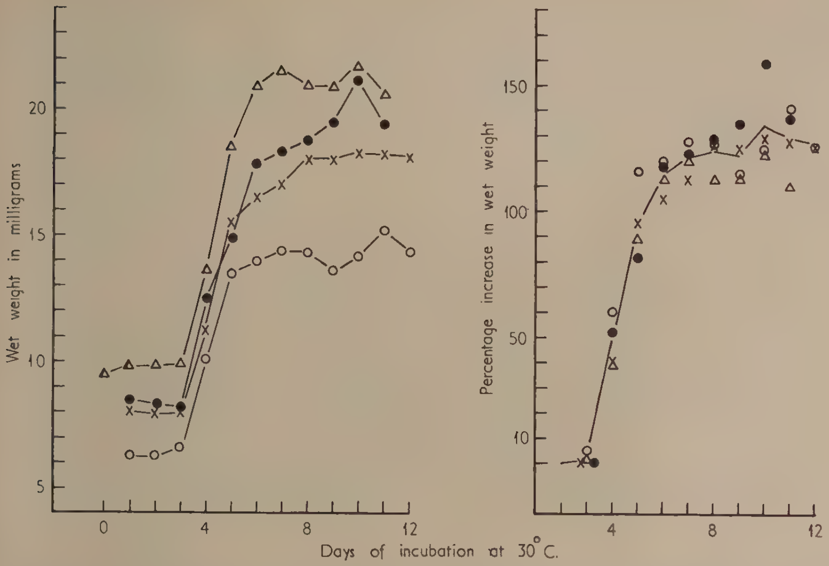
1. *Wet weight*

(*a*) *Eggs.* The results for four egg-pods are shown in Text-fig. 2. The second graph in Text-fig. 2 shows the same results expressed as percentages of their respective weights at one day.

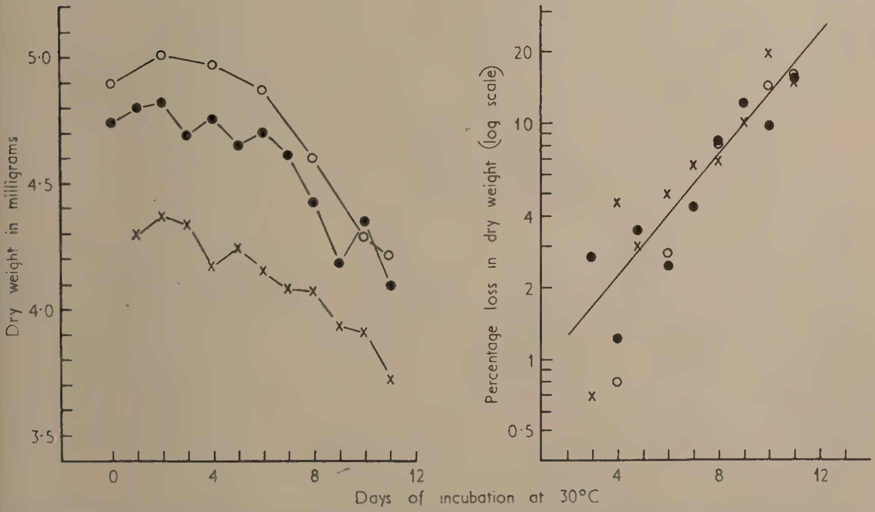
(*b*) *Embryos.* The wet weight of embryos, estimated from R.W. and density measurements, is included in the discussion section.

2. *Dry weight*

(*a*) *Eggs.* The results of three sets of measurements are shown in Text-fig. 3. The second graph in Text-fig. 3 presents the decreases in dry weight as percentages of the maximum weight recorded for each set of measurements (in all

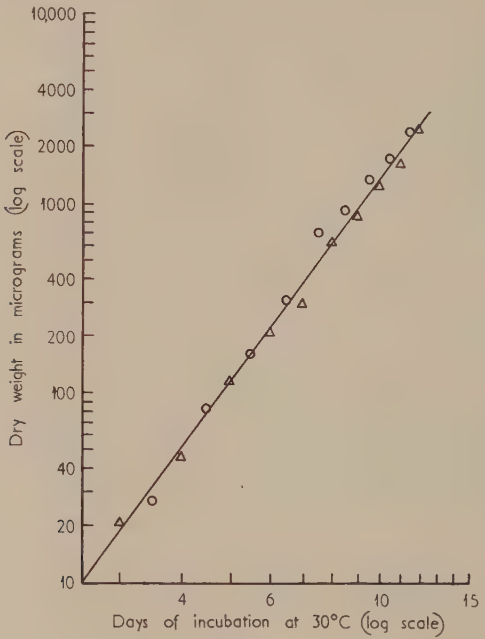


TEXT-FIG. 2. Wet weight of egg. Each symbol refers to eggs from a single pod, and each point is the mean value for six eggs weighed singly. The curve for the percentage increase in wet weight has been drawn through the means of the four sets of measurements.



TEXT-FIG. 3. Dry weight of egg. Symbols ○ and ● refer to eggs from single pods, each point being the mean of six eggs weighed singly. Symbol × refers to eggs from seven pods, each point being the mean of 12–24 eggs weighed singly. The curve for the percentage loss in dry weight has been fitted to the means of the three sets of measurements.

cases the maximum weight was recorded on the second day). The graph is a semilogarithmic plot and the fitted straight line represents an equation of the form $\log_{10} Y = kt + C$, where Y is the percentage loss in weight, t is the number of days of incubation, k is the relative rate of loss with a value of 0·129, and C is a constant equal to —0·1607.

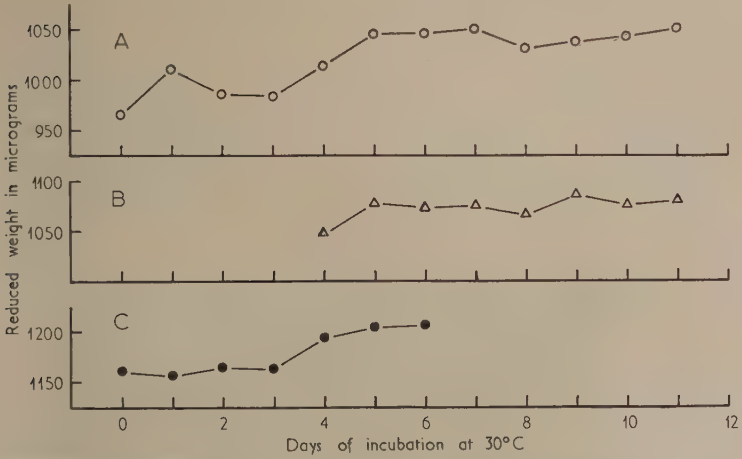


TEXT-FIG. 4. Dry weight of embryo. Each symbol refers to embryos from six egg-pods, and each point is estimated from 1–9 batches of from 50 embryos to 1 embryo per batch according to their age. The curve has been fitted to the combined two sets of measurements.

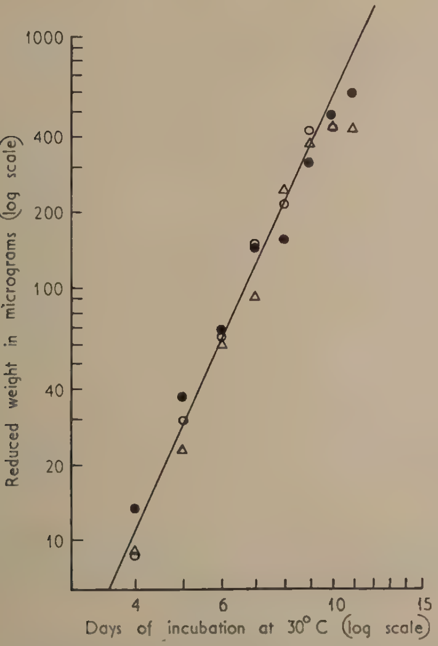
(*b*) *Embryos.* Text-fig. 4 shows the results of two sets of measurements, plotted on a double logarithmic scale, i.e. $\log_{10}$ dry weight against $\log_{10}$ number of days of incubation. The straight line represents the best fitting equation of the form $\log_{10} W = k \log_{10} t + C$, where W is the dry weight in micrograms (μg.), t is the number of days of incubation at 30° C., k is the relative growth constant equal to 3·578, and C is a constant equal to —0·4436.

3. *Reduced weight*

(*a*) *Eggs.* Text-fig. 5 shows the results of three sets of measurements. The same eggs were weighed each day and the handling and exposure to lower temperature tended to delay development, particularly in the case of those used for the first 4 days of development. The whole incubation period has been



TEXT-FIG. 5. Reduced weight of egg. Each symbol refers to eggs from a single pod, the same eggs being used every day. ○ refers to 1, △ to 12, and ● to 11 eggs.



TEXT-FIG. 6. Reduced weight of embryo. Each symbol refers to embryos from a single pod, each point being the mean of six embryos. The curve has been fitted to the means of the three sets of measurements for the fourth to ninth days.

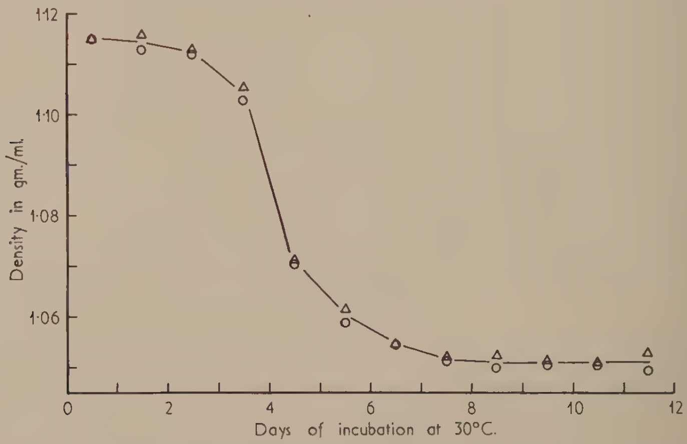
covered by two sets of eggs with an overlap on the fourth, fifth, and sixth days, and by one egg for the whole period of incubation.

(*b*) *Embryos.* The results of three sets of measurements are given in Text-fig. 6. The data have been plotted on a double logarithmic scale and, as in the case of the dry weight graph, the straight line in the figure represents an equation of the form $\log_{10}$ R.W. $= k \log_{10} t + C$. This has been fitted by the method of least squares to the values for the fourth to ninth days only since subsequent values seem to depart from the relationship. In this equation k is 4·345 and C is —1·5800.

In one set of measurements, on the eleventh day, the egg, the intact embryo, the isolated mid-gut containing the yolk, and the egg-shell were weighed, as well as the embryo free from yolk. Their mean values for four eggs were 851, 820, 63, 28, and 593 μg. respectively. The reduced weight of the embryo should therefore have been 820—63 = 757 μg. and not 593 μg. It would seem that there had been considerable loss of embryonic material when removing the yolk-filled mid-gut, and it is likely that such losses account for the departure from the double logarithmic relationship after the ninth day.

4. *Density*

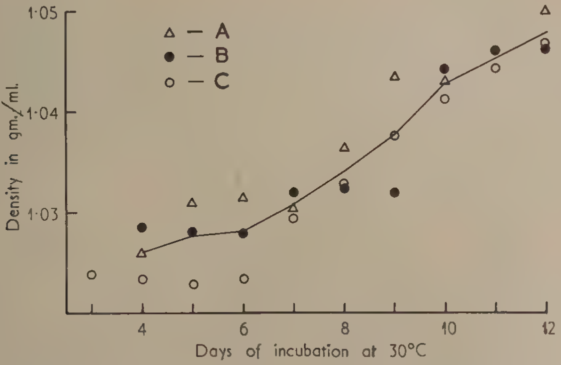
(*a*) *Eggs.* Text-fig. 7 shows the results for two pods.



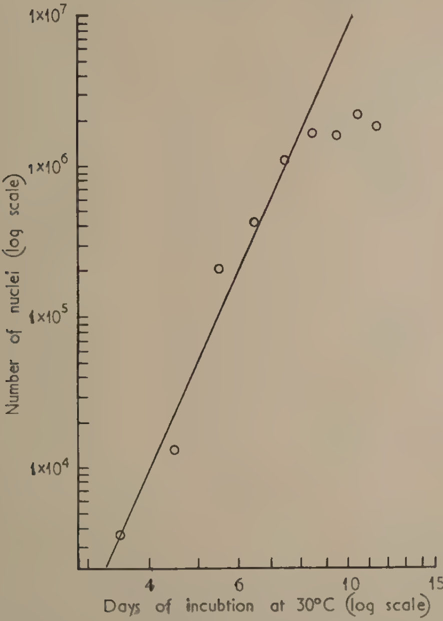
TEXT-FIG. 7. Density of egg. Each symbol refers to eggs from a single pod, and each point is the mean of six eggs measured singly. The curve has been drawn through the means of the two sets of measurements.

(*b*) *Embryos.* Text-fig. 8 shows the results for three series of measurements. Two pods were used in each of series A and C, and one pod was used for series B. The curve drawn in Text-fig. 8 is the mean of the three sets of measurements. The results for series A and B were somewhat irregular but the density change recorded for series C agrees quite well with the mean curve for the three pods.

The mean curve is therefore the best estimate of the density of the embryo that is available.



TEXT-FIG. 8. Density of embryo. Series *A* and *C* each refer to embryos from two pods and *B* refers to a single pod. Each point is the mean of 2–12 (usually about six) embryos measured singly. The curve has been drawn through the means of the three sets of measurements.



TEXT-FIG. 9. Number of nuclei per embryo. The results are for eggs from a single pod, each point being the mean of 3–6 embryos measured singly. The curve has been fitted to the means for 3½ to 8½ days.

5. *Nuclear number*

(*a*) *Eggs.* No measurements.

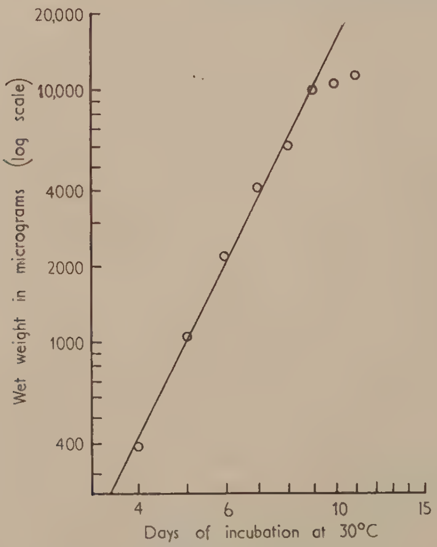
(*b*) *Embryos.* Text-fig. 9 shows the results of a single series of measurements using one pod. The results are too few and varied to indicate the precise nature of the change in rate of cell-division, but in conformity with the rest of the growth data they have been plotted as a double logarithmic function. An equation of the form $\log_{10}$ number of nuclei $= k \log_{10} t + C$ has been fitted to the data from $3\frac{1}{2}$ to $8\frac{1}{2}$ days for which k is 7·344 and C is $-0{\cdot}4318$. Nuclear number appeared to remain constant at about 2×10^6 after the ninth day.

DISCUSSION

The growth of the locust embryo

Three measures of the growth of the embryo have been made—dry weight, reduced weight, and number of nuclei. A fourth measure, the wet weight, may be estimated by combining the reduced weight data with those for the density of the embryo, using the formula

$$\text{Wet weight} = \frac{\text{Reduced weight}}{1 - \dfrac{\text{Density of water at } 20^\circ\text{ C.}}{\text{Density of embryo}}}.$$



TEXT-FIG. 10. Estimated wet weight of embryo. The points have been estimated from the mean R.W. and density values using the relationship

$$\text{W.W.} = \frac{\text{R.W.}}{1 - \dfrac{\rho\text{W}}{\rho\text{E}}}.$$

The curve has been fitted from the fourth to ninth days.

This calculation has been done using the mean measured R.W. and density values and the result is shown in Text-fig. 10. This is a double logarithmic plot and the straight line represents the fitted equation of the usual form with k equal to 3·937 and C equal to 0·2516 for the fourth to ninth days.

All four measures of growth have been summarized by equations of the form $\log_{10} Y = k \log_{10} t + C$, where Y is the quantity measured, t is the age of the embryo in days, and k is the growth constant. The data for the dry weight can be fitted equally well, judged by the R value of Yule & Kendall (1950), by two simple exponential equations of the form $\log_{10} Y = kt + C$ in the manner of Brody (1927, 1945) with k equal to 0·305 for the third to eighth days and 0·133 for the end of the eighth day to the end of the twelfth day of incubation. Since the other measures are best summarized by the double logarithmic relationship, this has been preferred for the dry weight also and permits easy comparison of the different measures. The double logarithmic relationship is the one commonly used to describe the growth of vertebrate embryos where, as has been noted by Hayes (1949), the constant k is often of the order of 2 or 3. The values of k for dry weight (3·6), wet weight (3·9), and reduced weight (4·3) of locust embryos may seem high. The vertebrate values usually refer to the modified equation of MacDowell, Allen, & MacDowell (1927), i.e. $\log_{10} W = k \log_{10} (t - n) + C$, where n is the time from fertilization to the establishment of the embryonic axis. The nearest equivalent to the embryonic axis in the locust egg may be taken to be the 'primary epithelium with embryonic and extra-embryonic regions' described by Roonwal (1936*b*) as appearing after 28 hours of incubation. If, therefore, n is taken as one day for the locust and the values of k are recalculated they become 3·0 for dry weight, 3·3 for wet weight, and 3·6 for reduced weight. The growth constants of the locust embryo would appear to be similar to those of various vertebrate embryos such as those given by MacDowell, Allen, & MacDowell (1927), Murray (1925), and Byerly (1932).

It must be remembered that the growth equation was fitted from the third to the twelfth days for dry weight, from the fourth to ninth days for R.W. and wet weight, and from $3\frac{1}{2}$ to $8\frac{1}{2}$ days for nuclear number. There were no measurements prior to the third or fourth days. In the case of R.W. and wet weight the measured values for the tenth to twelfth days were lower than expected by extrapolation from the equations. At the end of the eleventh day the growth equation would predict a R.W. of 880 μg. and a wet weight of 22·5 mg. The equivalent experimental means for the eleventh day were 514 μg. and 11·4 mg. respectively. Part of this discrepancy in weight may be due to losses of embryonic material incurred when removing the yolk which becomes enclosed by the embryo by the end of the eighth day. In the case of the R.W. measurements the yolk was washed out with a pipette, and this may have swept out embryonic material too. (In the other experiments the yolk was carefully squeezed out of a small slit in the dorsal surface of the embryo. This disturbed the contents of the embryo less and may account for the fit of the dry weight values right up to the twelfth day.) Furthermore, since the older embryos had to be opened in order to remove the yolk, the density measurements refer to the embryonic tissues rather than to the embryo with its cavities and their contents. Thus the calculated wet weight values will be underestimates of the true wet weight and probably refer to the embryonic tissues alone. In spite of this it must also be recognized that there is the possibility of a departure from the growth equation towards the end of the incubation period, for the predicted R.W. of 1,285 μg. by the end of the twelfth day exceeds the average R.W. of the intact egg, which was about 1,100 μg. and ranged from 1,000 to 1,200 μg. for different pods. The same is true for wet weight where the predicted twelfth-day value is 31·6 mg. and egg weights ranged from 14 to 22 mg.

Nuclear number seemed to increase more rapidly than any of the other growth measures, but the growth curve is a poor fit and it is not certain that increase in number of nuclei followed the same pattern as the increase in embryonic material. Furthermore, either cell-division ceased before the end of the ninth day or, owing to cellular differentiation and cuticle formation, nuclear isolation becomes increasingly difficult. In the first case there is an upper limit of $1\frac{1}{2}$–2 million nuclei in the locust embryo. If growth continued according to the fitted equation then there would be about thirty million nuclei by the end of the twelfth day. It seems unlikely that a difference of this order would fail to be detected even by the crude maceration technique that was used. In a similar set of measurements on post-quiescent eggs of *Locustana pardalina* (Walk) I found that nuclear number increase began 3 days after wetting the eggs, at 30° C., and rose from 4×10^5 to approximately $2{\cdot}2 \times 10^6$ by the end of the eighth day, i.e. 3 days before hatching. Again there seemed to be an upper limit of the order of two million nuclei. In studying the time of activation of embryonic endocrine glands in the same species Jones (1956) looked for mitotic figures and found them only from the third to the eighth days. From the reviews of methods of isolating nuclei by Dounce (1952) and Schneider & Hogeboom (1951) it would appear that citric acid gives good recovery of nuclei in good physical condition. In an attempt to test the efficiency of my nuclear number technique I estimated the number of nuclei in an embryo from sample counts on a selected series of sections. The embryo was from a post-quiescent egg of *L. pardalina* (Walk) that had been incubated for 3 days. Using the corrections described by Abercrombie (1946) a total of 313,146 was obtained. Four similar embryos used in the maceration technique gave a mean count of 400,000 with a standard error of the mean of 60,000. The two methods agree reasonably well.

The values of k for the four measures of growth may be compared directly. Thus the t-test described by Bailey (1959) shows that the k's for wet and dry weights are not significantly different, whereas those for R.W. and nuclear number differ from that of dry weight at the 0·01 level of significance. Alternatively the relative growth rates may be examined by means of double logarithmic plots in the manner of Needham (1942). A better method is to combine the separate growth measures to give new measures which reflect their relationships.

Three such measures are shown in Table 3. The percentage water content of the embryo reflects both the dry and wet weight changes. The values given in Table 3 suggest that there were no great differences in water content except perhaps a tendency for it to decrease after the ninth day. Reduced weight is a measure of the amount of embryonic material of density different from that of water and might be expected to be consistently related to the dry weight provided that the mean density of the dry embryonic material does not change. This density may be calculated using the relation

TABLE 3

Relations of wet weight, R.W., and nuclear number to dry weight of embryo

Days of incubation at 30° C.	*Water content of embryo (percentage)*	*Density of dry embryo*	*Dry weight per nucleus*
		(g./ml.)	(μg.)
3½	—	—	75×10^{-4}
4	88·0	1·29	—
4½	—	—	64×10^{-4}
5	89·1	1·35	—
5½	—	—	$7\cdot5\times10^{-4}$
6	90·6	1·44	—
6½	—	—	$7\cdot3\times10^{-4}$
7	92·9	1·79	—
7½	—	—	$6\cdot3\times10^{-4}$
8	89·6	1·50	—
8½	—	—	$5\cdot8\times10^{-4}$
9	91·4	1·79	—
9½	—	—	$8\cdot2\times10^{-4}$
10	88·6	1·60	—
10½	—	—	$7\cdot8\times10^{-4}$
11	86·1	1·48	—
11½	—	—	$13\cdot3\times10^{-4}$

$$\text{Density of dry embryo } (\rho\,\text{D.E.}) = \frac{\text{Dry weight of embryo (D.W.)} \times \text{Density of water at } 20^\circ\text{ C. } (\rho\, W)}{\text{Dry weight of embryo (D.W.)} - \text{Reduced weight of embryo (R.W.)}}$$

The results of this calculation using the mean R.W. and dry weight results are given in Table 3. It can be seen that the density of the dry embryo rose at first and then fell. However, this calculation is valid only if the dry weight refers to the same material as the R.W. It is possible that the drying technique caused some destruction of material, for example by driving off bound water. The fact that the densities in Table 3 are higher than that of protein (1·27 g./ml.) suggests that the dry-weight figures are indeed too low in comparison with the R.W. figures. The third measure in Table 3, the dry weight per nucleus, has been calculated from the dry weight and nuclear number results. The ratio of these measures decreased rapidly to a low level, confirming that cell-division occurred at a higher rate than the formation of new protoplasm. The possible slight increase after the ninth day corresponds with the cessation of nuclear number increase and could represent the enlargement of cytoplasmic structures involved in differentiation of the embryo, e.g. muscle and cuticle formation. The only other information on nuclear number is that of Sze (1953) for embryos of *Rana pipiens* where the increase was described by two exponential equations, one covering the period before gastrulation with a growth constant of 0·18 and the other with a constant of 0·0067 operating from gastrulation to the 200th hour. Work on mitotic indices reviewed by Bragg (1938) has also shown that the rate of cell-division may show a decrease soon after the beginning of gastrulation in several vertebrate embryos. He reported a decrease in cell multiplication at gastrulation for *Bufo cognatus*. Derrick (1937) reported the same in the case of chick embryos, and Jones (1939), working on the mitotic index of *Fundulus heteroclitus* embryos, referred to reports of a drop in mitotic index at gastrulation. It would appear that in general nuclear number increase does not necessarily correspond with growth in dry or wet weight.

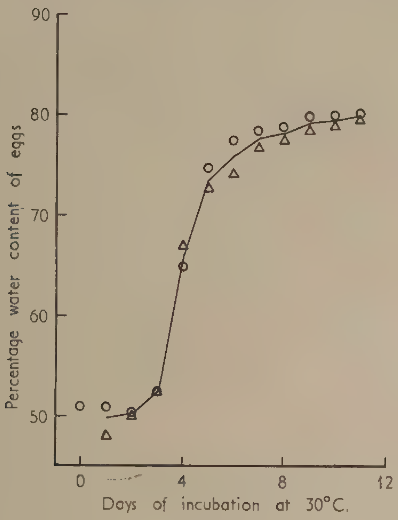
Efficiency of growth

The results for the dry weight of the whole egg have shown that the loss in dry material may amount to 20–25 per cent. by the end of the twelfth day of incubation. This agrees with the loss of 20 per cent. recorded by Roonwal (1936*a*) but not with the figure of 12 per cent. given by Shulov & Pener (1959) for the same species. The difference in the latter case may be connected with the different incubation temperatures and times; cf. Gray's (1928) report that more yolk is required for maintenance of the trout embryo at higher than at lower temperatures. Johnson (1937) gave figures for *Notostira* which indicate a loss of 15–25 per cent., and Rainey (1950) reported a loss of 14–22 per cent. in the eggs of *Lucilia*. Thus a loss of 20 per cent. seems typical of many insect eggs, at normal developmental temperatures.

The semi-logarithmic plot of the mean percentage loss in dry weight of the egg against time of incubation shown in Text-fig. 3 has a regression coefficient of 0·129. This suggests that the changes in rate of respiratory loss in dry weight matched the changes in rate of increase of the dry weight of the embryo after the eighth day ($k = 0{\cdot}133$ for the simple exponential relationship of embryonic dry weight) but that the latter rate changed more rapidly before this time ($k = 0{\cdot}305$ for the simple exponential equation of embryonic dry weight). This difference could be associated with the relatively greater importance of extra-embryonic structures and their activities during early development, for they contributed to the losses in dry weight of the egg but not to the increases in dry weight of the embryo. Bodine & Boell (1936) have shown that in the grasshopper the yolk and extra-embryonic tissues make a considerable contribution to the respiration rate. Losses incurred in the drying process in the present work would also have given over-estimates of the dry weight losses which would be particularly noticeable early in development when the true respiratory losses are small.

The water relationships of the locust egg

Brief reviews by Wigglesworth (1953), Needham (1942), and Slifer (1938) have shown that increase in weight due to the absorption of water is a feature common to many insect eggs. The curve of increase in wet weight for the locust is similar to those obtained for many other insects. In particular, the percentage increase curve of Text-fig. 2 is easily compared with similar curves given by Johnson (1937) for *Notostira erratica* L., and by Kerenski (1930) for *Anisoplia austriaca* Reitt. Typically there is a preliminary phase of constant weight, then a phase of rapid increase, and finally a period of more or less constant weight. The degree of this increase is also typical, being usually well over 100 per cent. (120 per cent. for *Anisoplia, Notostira,* and *Locusta*). In a recent paper, Shulov & Pener (1959) reported the results of wet and dry weight measurements for the same species but incubated at 27° C. and apparently using batches of egg-pods. Development took 16 days and weight increased by 126 per cent., but this increase was spread over 6 days (second to eighth). They gave the same increase for *Schistocerca*



TEXT-FIG. 11. Percentage water content of egg. Symbol △ refers to eggs from seven pods, each point being the mean of 12–24 eggs weighed singly, and ○ refers to eggs from a single pod, each point being the mean of six eggs weighed singly. The curve has been drawn through the means of the two sets of measurements.

gregaria (Forsk.). In the present work in two sets of measurements the wet and dry weights of the same eggs were determined and so the percentage water content of the locust egg may be calculated. As can be seen from Text-fig. 11, this paralleled the changes in wet weight until the later stages of development when, owing to the increasing magnitude of the dry weight loss, the percentage of water content showed a slight increase and the wet weight tended to become steady. For the first 3 days the water content was about 50 per cent., rose rapidly in the next 2 days to 70–75 per cent., and then increased more slowly to reach about 80 per cent. by the end of the eleventh day. Roonwal (1936*a*) measured the wet and dry weight of the eggs of *Locusta migratoria* at 0, 6, and 13 days and found the percentage water content to be 51·92, 77·8, and 82·36 respectively. Ludwig (1932) recorded an increase from 49 per cent. to 80 per cent. for *Popillia japonica* Newman, Johnson (1937) found a rise from 46·85 per cent. to 74·75 per cent. in the case of *Notostira,* and Laughlin (1957) gives figures of 50 per cent. rising to 80 per cent. with very little variation for *Phyllopertha horticola* L.

Changes in the density of the locust egg (Text-fig. 7) showed an inverse relation to the changes in water content, i.e. the density reflected the water content of the egg. In the egg of *Rana pipiens,* Briggs (1939) found that the density reflected the water uptake and the formation and subsequent collapse of the blastocoel and archenteron. Similar results for *Xenopus laevis* have been obtained by Tuft (1957) who has used density and R.W. measurements to show that water collects first in the blastocoel and archenteron and, after the collapse of these, in the embryonic cells. In general it seems that movements of water are sufficient to mask any possible effects on the density of the egg of other embryonic events, such as transformation of yolk into embryo. In the present work, the wet weight as well as the density was measured, and so direct comparison of water uptake and density change may be made. If the density of the egg at $\frac{1}{2}$ day is taken as correct, then the changes in density due to water intake may be calculated as in Table 4 for the two pods, both with an average weight of 8 mg. for the fresh-laid egg. It can be seen from the Table that the calculated densities are lower than the measured values by about 0·006 g./ml. after the fourth day, i.e. once water uptake begins. This discrepancy could be due to changes in the density or amount of dry material in the egg. Changes of this kind should also be recorded in the R.W. measurements. It has already been shown (Text-fig. 5) that the R.W. of the egg increased by about 40 μg. during the fourth and fifth days, but that apart from this there was no change. This is surprising in view of the loss of up to 20 per cent. in dry weight (Text-fig. 3) and can only be explained in terms of an increase in the density of the dry material of the egg. This density change is the inverse of the change in dry weight of the egg (subject to losses due to the drying procedure). It is possible therefore that it is due to the selective combustion of fat (density 0·8 g./ml.) but might also involve other processes such as the chemical combination of water with the embryonic material. Slifer (1930) found that fatty acids formed 17–22 per cent. of the dry weight of the grasshopper egg and that more than 50 per cent. of this was lost during development. The work of Boell (1935) on respiratory quotients of the eggs of grasshoppers has indicated the importance of fat in respiration, and in a review (1955) of energy sources in insect eggs he has suggested that fat is probably the chief source in silkworm and grasshopper eggs. Wigglesworth (1953) also quoted work on the silkworm and on *Dixippus* showing that in both cases fat yielded two-thirds of the energy and decreased by half during development. Protein combustion will tend to counteract the density effect of the fat consumption, and Bodine (1946) showed that uric acid formation in the embryo of *Melanoplus* paralleled the respiratory curve.

TABLE 4

Density of eggs in g./ml. Measured values compared with values computed from the wet weight (assuming that an 8-mg. egg has a density of 1·115 g./ml.)

Days of incubation at 30° C.	*First pod*			*Second pod*		
	Measured density	*Calculated density*	*Difference*	*Measured density*	*Calculated density*	*Difference*
½	1·1150	1·1119	−0·0031	1·1150	1·1119	−0·0031
1½	1·1155	1·1150	−0·0005	1·1130	1·1134	+0·0004
2½	1·1130	1·1150	+0·0020	1·1120	1·1119	−0·0001
3½	1·1055	1·1033	−0·0022	1·1030	1·1008	−0·0022
4½	1·0710	1·0648	−0·0062	1·0705	1·0676	−0·0029
5½	1·0615	1·0560	−0·0055	1·0590	1·0534	−0·0046
6½	1·0545	1·0497	−0·0048	1·0545	1·0503	−0·0042
7½	1·0520	1·0469	−0·0051	1·0515	1·0467	−0·0048
8½	1·0525	1·0459	−0·0066	1·0500	1·0445	−0·0055
9½	1·0515	1·0461	−0·0054	1·0505	1·0433	−0·0072
10½	1·0510	1·0438	−0·0072	1·0505	1·0453	−0·0052
11½	1·0530	1·0469	−0·0061	1·0495	1·0445	−0·0050
12½	1·0485	1·0431	−0·0054	—	—	—

There was a localized increase of 40 μg. in the R.W. of the egg. It is significant that this increase occurred during the 2 days of maximum rate of water uptake (fourth and fifth days, Text-fig. 2). Both this increase in R.W. and the discrepancy between the measured and calculated egg densities can be explained by assuming the presence of air in the chorion of the fresh-laid egg and that this air is displaced by the incoming water. The volume of this air may be calculated using the relationship R.W. = W.W.—V_{pw} and typical values for R.W., wet weight, and density of water at 30° C. (0·998203 g./ml.). Thus with air present, i.e. as measured, 1,060 = 8,000—V_1 0·998203 and therefore V_1 = 6·952 μl. If air were not present then the R.W. would be 40 μg. higher and so 1,100 = 8,000—V_2 0·998203 and V_2 = 6·912 μl. The volume of air is therefore 6·952—6·912 = 0·040 μl. This quantity of air will account for the discrepancy in densities. Thus an 8-mg. egg with density of 1·1150 g./ml. has a volume of 8/1·115 μl., i.e. 7·175 μl. If 0·04 μl. of air were not present then the volume would be 7·175—0·04, i.e. 7·135 μl. and the density would be 8/7·135 or 1·1212 g./ml. This is 0·0062 greater than 1·1150 g./ml., and so the discrepancy between calculated and measured densities (0·006) is fully accounted for. Roonwal (1936*a*) failed to detect air spaces in the locust egg by microscopical examination. Tuft (1949) has demonstrated the presence of air under the chorion of the egg of *Rhodnius prolixus*. The presence of air in or under the chorion has been shown for the locust and other insects by Wigglesworth & Beament (1950) and Wigglesworth (1953). In many eggs the air is present throughout the development of the egg while in some it only appears later in development. It is interesting, therefore, that Wigglesworth and Beament could detect air in the locust egg only in the dry chorion.

It would seem that changes in water content are responsible for all the observed density changes in the egg with the dry weight loss as an obvious factor in the changing water content after the fifth day. The sudden intake of all the water required for development is peculiar to the partially cleidoic terrestrial egg. In the eggs of *Sepia,* Ranzi (1929) has shown that the water intake occurs throughout development presumably as it is required for building the embryo. The ability to absorb and store all the required water during a short period whenever the water is available would be of species survival value. The next evolutionary step is that of the completely cleidoic egg in which all the required water is contained within the egg before laying. A recent study by Roth & Willis (1955) of the water content of cockroach eggs showed a series of adaptations paralleling the evolutionary steps towards complete independence from an external water supply.

SUMMARY

1. Three measures of the growth of the embryo of *Locusta migratoria migratorioides* (R. & F.) were determined from the third to the twelfth days of development at 30° C. These measures were the dry weight, reduced weight, and number of nuclei. Measurements of the density of the embryo were also made and used with the R.W. to calculate the wet weight. The same measurements of the intact egg were taken throughout development.

2. The growth in dry weight of the locust embryo from the third to twelfth days of development appeared closely comparable with that of vertebrate embryos such as the chick embryo and could be described by the usual double logarithmic relation with growth constants of closely comparable values.

3. Increase in R.W. and wet weight could be similarly described from the third to ninth days after which the rate of increase fell away more rapidly than expected.

4. Increase in nuclear number occurred at a higher rate than any of the other measures up to the ninth day after which no further increase above a total of about two million nuclei could be detected. As in some vertebrate embryos, cell multiplication seemed not to parallel growth in weight.

5. The implications of the differences in rates of increase of these measures were considered. From the third to ninth days the embryo was approximately 90 per cent. water, the density of the dry material may have increased and the dry weight per nucleus decreased rapidly to a constant value. After the ninth day there appeared to be a fall in water content and dry density and an increase in the dry weight/nuclear number ratio. The density of the wet embryo increased steadily after the sixth day.

6. The total loss in dry weight of the egg due to respiration was about 20 per cent., an amount typical of many insect eggs.

7. The eggs increased in weight by about 120 per cent., chiefly during the fourth and fifth days. The percentage water content rose from about 50 per cent. to about 80 per cent. Changes in the density of the eggs reflected perfectly the changes in water content.

8. The R.W. of the eggs was constant except for a rise of about 40 μg. during the fourth and fifth days. It was suggested that this could be accounted for by the presence in the chorion of about 0·04 μl. of air which was displaced when water uptake took place after the third day. This would also have accounted for the very small difference in the measured changes in density compared with those calculated from the water intake. A constant R.W. and a large decrease in dry weight imply that the density of the dry material of the eggs must have increased at the same rate as the respiratory loss in dry weight. Combustion of fat might account for this.

RÉSUMÉ

La croissance de l'embryon de Sauterelle

1. La croissance de l'embryon de *Locusta migratoria migratorioides* (R. et F.) a été étudiée par trois procédés de mesure entre le 3ème et le 12ème jour du développement à 30° C. Ces trois procédés sont les mesures du poids sec, du poids réduit (P.R.) et du nombre de noyaux. La densité de l'embryon a été aussi mesurée et utilisée concurremment avec le P.R. pour calculer le poids frais. Les mêmes mesures ont été faites sur l'œuf intact au cours du développement.

2. L'accroissement du poids sec de l'embryon de Sauterelle entre le 3ème et le 12ème jour de développement paraît tout à fait comparable à celui des embryons de Vertébrés, tel l'embryon de Poulet. Il peut être exprimé par la double relation logarithmique usuelle avec des constantes de croissance de valeurs très comparables.

3. L'accroissement du P.R. et du poids frais peut aussi être représenté de la même manière du 3ème au 9ème jour, après quoi le taux de croissance diminue beaucoup plus rapidement qu'on pourrait l'attendre.

4. L'accroissement du nombre des noyaux se produit jusqu'au 9ème jour à un taux plus élevé qu'aucune des autres mesures, après quoi on n'a pu mettre en évidence aucune augmentation ultérieure au delà du total d'environ deux

millions de noyaux. Comme chez certains embryons de Vertébrés, la multiplication cellulaire ne semble pas être parallèle à la croissance en poids.

5. Quelle est la signification des différences entre les taux de croissance de ces mesures? Du 3ème au 9ème jour, l'embryon contient à peu près 90 pour cent d'eau, la densité du matériel sec peut avoir augmenté et le poids sec par noyau peut avoir diminué rapidement jusqu'à une valeur constante. Après le 9ème jour, il semble y avoir une chute de la teneur en eau et de la densité du matériel sec, et une augmentation du rapport poids sec/nombre des noyaux. La densité de l'embryon frais augmente constamment après le 6ème jour.

6. La perte totale de poids sec de l'œuf, due à la respiration, est d'environ 20 pour cent, proportion typique pour beaucoup d'œufs d'insectes.

7. L'augmentation de poids des œufs est d'environ 120 pour cent, principalement pendant le 4ème et le 5ème jour. Le pourcentage de la teneur en eau passe d'environ 50 pour cent à 80 pour cent. Les changements de densité des œufs reflètent parfaitement les changements dans la teneur en eau.

8. Le P.R. des œufs est constant, à l'exception d'une augmentation d'environ 40 μg. pendant les 4ème et 5ème jours. On suppose que ceci peut être dû à la présence dans le chorion d'environ 0·04 μl. d'air, qui est déplacé quand intervient l'absorption d'eau après le 3ème jour. Cela peut aussi rendre compte de la très petite différence dans les mesures des variations de densité, comparativement à celles que donnent les calculs de l'absorption d'eau. Un P.R. constant et une forte diminution du poids sec impliquent que la densité de la substance sèche des œufs doit avoir augmenté à mesure que le poids sec diminue du fait de la respiration. Ce phénomène peut s'expliquer par la combustion des graisses.

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Cortical Grafting in *Xenopus laevis*

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WITH ONE PLATE

INTRODUCTION

THE amphibian egg bears a cortex as the periphery of its cytoplasm. The interpretation by Dalcq & Pasteels (1937) of experiments performed by Pasteels (1938, 1940, 1941), in which fertile eggs of *Rana fusca* were centrifuged or inverted, led to the suggestion that the cortex possesses properties which control the course of development to some degree. It appeared that the properties of the cortex of a fertile egg were such that a particular portion of it, that lying in the grey crescent region, was involved in the determination of the site of the dorsal lip when gastrulation occurred and thus of the general axiality of the embryo. In these experiments the relation between the cortex and the underlying cytoplasm was disturbed and it was possible to alter the natural relations of any given piece of the cortex with the cytoplasm beneath. But it was not possible to alter the relationship of one part of the cortex to another. In consequence it was impossible to perform any experiment which might reveal how the properties of the cortex are organized within itself. Moreover, since it was impossible to move a piece of cortex from one embryo to another, it could not be denied that the structure of morphogenetic importance which was resistant to centrifugation or inversion might be other than the cortex. Lehmann (1945) suggested the subcortical marginal plasm, though the experiments of Pasteels (1946) make this suggestion improbable. Grafting the cortex from one embryo to another allows a test of this point as well as permitting investigation into the organization of any morphogenetic properties which the cortex may have: such an organization was suggested by Dalcq (1938).

MATERIALS AND METHODS

The eggs, fertile or unfertilized, were stripped of their membranes by hand and stored in a normal Holtfreter solution buffered to pH 6·2 with a 0·001 M 2-amino-2-hydroxymethyl-1,3-propanediol-sodium dihydrogen phosphate buffer.

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Hereafter this buffer system will be called a tris-phosphate buffer. Donor embryos and those receiving grafts were either uncleaved eggs or eggs in the midst of their first cleavage. Four or five minutes before grafting the donor embryo was moved to a 0·002 M solution of the chelating agent, ethylene-diamine tetra-acetate in calcium-free Holtfreter solution, buffered to pH 8·1 with 0·002 M 2-amino-2-hydroxymethyl-1,3-propanediol and hydrochloric acid. A minute before grafting the recipient embryo was placed in this solution next to the donor. After 4–5 minutes it was possible to lift off portions of the cortical structure using fine tungsten needles, leaving an undisturbed cytoplasm beneath. The grafts were usually square or oblong in shape, measuring about 150 μ each way. This size of graft represents about 5 per cent. of the whole cortical area. Using this method it has so far been impossible to remove pieces of cortex from regions near the vegetal pole. A fresh pair of needles was used for each graft. Six pieces of the cortex were fixed for electron microscopy using osmic fixation and embedded in Araldite by Mr. M. Kidd, who sectioned and examined them and to whom I am very grateful. The cortex removed in this manner varies from 0·5 to 3·0 μ in thickness; it contains a true cell surface at the periphery, a thin hyaline layer, and mitochondria, pigment granules, &c., within. Dollander (1951) described a similar structure for the cortex of various urodele eggs. It was, of course, impossible to decide whether these pieces corresponded exactly with that part of the cortex which has been shown to be resistant to centrifugation and inversion. But the stability and strength it possessed in comparison to the underlying cytoplasm make this very probable.

In order to insert the graft in the receiving embryo, a slight cut was made at the site for grafting; this cut slowly opened out. The graft was led into place with the needles and gently laid on the cut. Care was taken to ensure that it lay with its cell surface outermost and that no portion overlapped the surrounding cortex. To cause the graft to heal in, it was necessary to do no more than add sufficient calcium ion to the medium and to drop the pH enough for wound healing to take place. This was done by adding equal volumes of 0·03 M calcium chloride solution and normal Holtfreter solution buffered with tris-phosphate to pH 6·2. About 5 ml. of each were added to the Petri dish, which contained about 5 ml. of the solution of chelating agent. The medium was then slowly replaced with normal Holtfreter at pH 6·2 by repeated additions and removals. Healing was very rapid and if the graft was properly placed it appeared to merge in with the surrounding cortex after a few minutes. Grafts put in upside down were thrown out during the healing process. If the graft was noticeably either more or less pigmented than the surrounding cortex it was possible to locate it for the first day or so of subsequent development.

Control embryos were of two sorts: first, unoperated embryos which had been treated in the same solutions as the grafted embryos, and secondly sham-operated embryos in the midst of the first cleavage in which a cut in the cortex was made but which received no graft. Experimental embryos and controls were cultivated in glass dishes. A few cleavage stages after the operation the medium was replaced by a 0·5 to 0·6 strength Holtfreter solution buffered at pH 6·2 with a 0·0005 M tris-phosphate buffer. This medium also contained 0·5 ml. per litre of a supplementary medium, the addition of which appeared to help towards the healthy survival of the embryos, though this point has not been tested properly. The reason for the use of this solution is that the chelating agent will have removed other metallic ions as well as calcium from the embryo. To prepare this solution the following chemicals were dissolved in one litre of glass-distilled water and autoclaved:

	(g.)
Magnesium sulphate, heptahydrate	6·00
Ferric sulphate	0·60
Manganese sulphate	0·02
Zinc chloride	0·01
Copper sulphate, pentahydrate	0·005

Control embryos were transferred to this medium as well. This appeared to be the most satisfactory culture medium until the embryos had reached Nieuwkoop's stage 26. Most embryos were fixed at this stage but some were cultured for 5 days: they were transferred to 0·2 strength Holtfreter solution after stage 26. For these, as well as for those at stage 26, Smith's formol-bichromate reagent was used for fixation. They were embedded in celloidin-paraffin and sectioned at 8-μ thickness.

Grafting operations were carried out using material from various parts of the cortex, thus:

Animal pole placed at animal pole
„ „ grey crescent
„ „ vegetal pole
Grey crescent placed at grey crescent
„ „ mid-ventral margin
Mid-ventral margin placed at grey crescent.

Some grafts were taken from unfertilized eggs; these grafts were placed in embryos in the midst of their first cleavage. Grafts from fertile embryos were taken either from uncleaved eggs or from embryos in the midst of their first cleavage and were placed in embryos in the midst of their first cleavage.

RESULTS

Control embryos

The unoperated embryos (23) all developed normally to stage 26. Embryos which were sham-operated received wounds in one of four sites: at the animal pole, the grey crescent, the presumptive ventral margin, or the vegetal pole. Since the first cleavage was in progress these wounds were made, when necessary, not in the furrow but to the left or the right of it. Those with animal pole wounds (10 embryos) showed displacement of their first few cleavage planes, but thereafter developed normally save that the brain cavities were rather constricted and their walls thicker than usual. This displacement of the early cleavage planes was also noted in the embryos with wounds at other sites. The 9 embryos wounded at the vegetal pole and the 11 wounded at the ventral margin otherwise developed normally. Of the 13 embryos with grey crescent wounds, 11 developed normally, but 2 developed into the 'ring' type of embryo described by Schechtman (1942), in which invagination fails to occur properly. Since his 'ring' type embryos were produced by isolating the presumptive dorsal lip from the lateral marginal regions in the very early gastrula, it is possible that, in some manner, the wounding described here can leave the dorsal lip isolated.

Grafts using unfertile material

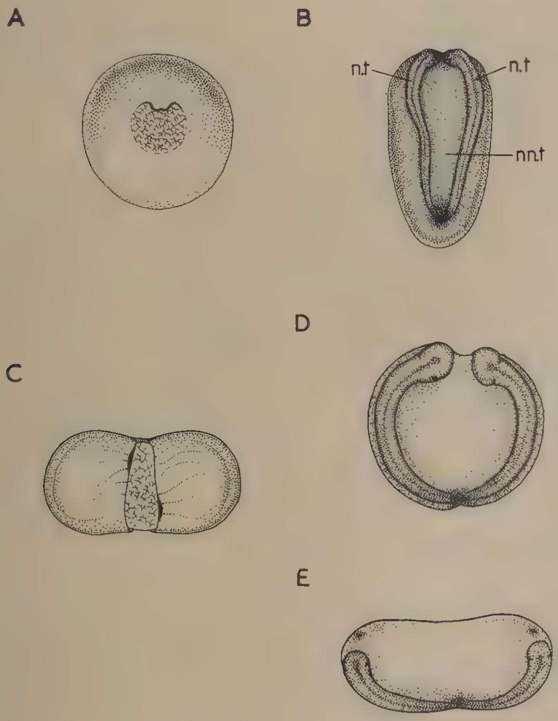
Two series of experiments, each with 8 embryos, were done. In the first, material slightly to the left of the animal pole was grafted to the same site; in the second it was grafted to the vegetal pole. In all cases the first cleavage, which was in progress in the receiving embryo, ceased and regressed. Grafts placed beside the animal pole allowed the appearance of furrows in the unpigmented part of the egg. A number of these, arranged irregularly, appeared, but it is uncertain whether they were cleavage furrows or not. In such embryos the pigmented part of the egg appeared to lie like a coherent cap on the remainder of the cytoplasm and never entered into this furrow formation. After some hours these processes stopped and the embryos became very fragile. Grafts placed at the vegetal pole also resulted in the cessation and regression of the cleavage furrow. In these embryos the pigmentation altered so that a broad equatorial band of pigment with lighter caps formed. The embryos gradually shaped themselves into fragile flattened balls.

Grafts using donor material of same age as receiving embryo

Each of the six types of graft mentioned earlier was taken from embryos in which the first cleavage was in progress. Since the first cleavage was in progress it was necessary both to take and to insert the grafts not medially but slightly to the right or to the left side of the embryo. After the grafts had healed in, the next few cleavage planes were often slightly abnormal in position. This abnormality was most pronounced on the side of the embryo to which the graft was made. Otherwise development appeared to be normal until the beginning of gastrulation.

When animal pole grafts were transplanted to the same site in a second embryo, gastrulation and neurulation took place normally in all the ten embryos used. However, subsequently, slight disturbances to the brain structure developed in four embryos, which appeared to be very similar to those in the control series.

Grafts of the animal pole material to the grey crescent region (9 in number) produced a typical situation at gastrulation: a central portion of the dorsal lip failed to form and invagination proceeded at two dorsal lips on either side of a slight protuberance. As a result two normal and nearly parallel axes were formed (Text-fig. 1 A, B; Plate, fig. D). The graft appears to form a slight amount of neural tissue composed of rather folded layers of cells.



Text-fig. 1. A, B, development of an embryo with animal pole material graft placed in the grey crescent. A, during gastrulation; B, after neurulation. *n.t.*, neural tissue, *nn.t.*, non-neural tissue. C, D, E, development of an embryo with grey crescent graft at ventral margin. In C embryo shows two neural plates and dorsal lips, one induced, the other normal. D, E show two extreme types of embryo which are produced by these grafts, after neurulation.

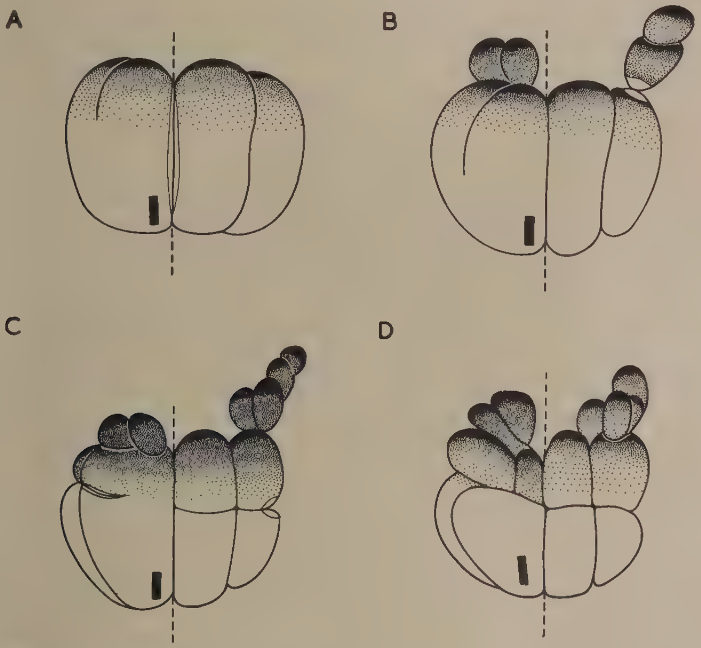
Animal pole material grafted at the vegetal pole was easily distinguished by its pigmentation. In embryos with these grafts the early stages of gastrulation proceeded normally. But after a while a very unusual form of exogastrulation took place. Instead of the exogastrulate being only a slumped heap of cells it was a coherent ball of cells which had a slightly pigmented surface. So far as could be seen it appeared that this pigmented surface contained the graft. A few loose cells lay around this ball. The balls appeared to contain about fifty cells apiece. Once this ball had left the embryo no further exogastrulation occurred. These balls could be separated from the embryos and cultured for several days, over which period they show no differentiation. Nine such embryos received this type of graft and behaved in this manner, and in all cases this exogastrulation removed so little material from the embryo that subsequent development was normal. Since exogastrulation occurred only twice in the other types of embryos and only to a very slight degree, it seems likely that it is a direct consequence of the insertion of the graft.

Grey crescent material grafted to its own site led to entirely normal development in 8 out of 11 embryos. The three that did not develop normally produced ring embryos similar to those found in the control group. When the grey crescent was transplanted to the ventral marginal region two dorsal blastopore lips were formed. One was in the normal position and the other at or near the site of the graft. These embryos (11) all produced these double dorsal lips which Pasteels (1941) was able to produce by suitable inversion of the egg. Invagination occurred at both lips, for a pair of axes were found in all these embryos (Plate, fig. A). These axes were arranged during early neurula stages so that their anterior ends pointed away from each other. During later stages they tended to become more parallel, as can be seen in Text-fig. 1 C, D, E, which show external views of such an embryo. Both axes are substantially complete, and two cross-sections of one of these embryos are shown in the Plate, figs. B, C.

Ten ventral margin grafts transplanted to the grey crescent region behaved in a manner rather similar to animal pole material at the same site. Gastrulation occurred on either side of the graft, leading to two entirely separate axes developed from the dorsal side of the blastopore. These axes were parallel in five cases, and at nearly parallel positions in the other five. The axes are similar in direction.

Grafts using donor material younger than receiving embryo

In order to test whether differences in age between donor and recipient influenced the experimental result, two sets of experiments were done. In the first, 5 embryos were given grafts at the vegetal pole, the material coming from the animal pole region of uncleaved fertile eggs. These embryos received their grafts while they were in the midst of their first cleavage. In the second, grey crescent material was placed in the ventral margin; the respective ages of graft and receiving embryo were the same as in the first. All these embryos showed a lag in cleavage in that half of the embryo which had the graft. This lag was from half to rather more than one cleavage stage behind the ungrafted half of the embryo. These effects are shown in Text-fig. 2. The discordance in cleavage age between the two sides of the embryo disappeared by about the sixth cleavage. Apart from these differences the embryos showed normal development until gastrulation began. Thereafter they behaved like similar grafts taken from embryos in the midst of their first cleavage, as described above.



TEXT-FIG. 2. Development (A–D) of an embryo which received, while it was in its first cleavage, a graft of animal pole material from an uncleaved fertile egg. This graft, placed at the ventral pole, is shown as a solid black oblong. Note the delay of cleavage on the side of the embryo with the graft. Dotted line indicates plane of first cleavage.

DISCUSSION

No profound effect on morphogenesis was produced by wounding the embryo, when this alone was done, as in the control series. There were, however, a small number of 'ring' embryos formed when the grey crescent was wounded. A few such embryos turned up in the experimental series when the operation was done at the same site, which suggests that they have a common cause. These 'ring' embryos are discussed below. The normal development of most of the control embryos suggests that slight wounding is not normally of noticeable morphogenetic effect. Likewise, those experiments in which a graft was made to the same type of site as that from which it was derived gave embryos which developed nearly normally. These may be regarded as forming part of the control experiments. At this point it should be remarked that the grafts do not replace portions of the cortex of the embryos in which they are placed; instead, they are additions to a complete cortex from which nothing is removed. These grafts do not represent more than about 5 per cent. of the surface area. In those embryos in which a 'control' operation was done it might have been expected that the added cortical material would result in the development of additional amounts of those organs in whose construction it was involved. But these grafts do not have such an effect, or at least any noticeable one. This argues against an extremely rigid control of morphogenesis in which each point of the cortex necessarily represents a certain future structure. This conclusion was clearly indicated by the work of Dalcq & Pasteels (1937), provided that it could be accepted that the cortex was the structure of morphogenetic importance which resisted centrifugation.

In designing the experiment to test the morphogenetic importance of the cortex it was decided to make those grafts which most closely resembled the experiments which led Dalcq & Pasteels (1937) and Pasteels, (1940, 1941) to propose the existence of a cortical field. By suitable centrifugation or inversion these authors were able to bring the junction of the light and heavy yolks close to and equidistant from the grey crescent in two places. Dorsal lips formed at both these sites, though neither lay at the site of the grey crescent. On this type of experiment in particular they based the idea that the dorsal lip of the blastopore and the subsequent formation of a nervous system, &c., was situated at a place determined by the interaction of two morphogenetic factors. One, the yolk gradient, was localized in the yolk, the other was present in the cortex. The ventral margin region is close to the junction between the two types of yolk. In consequence the grafting of grey crescent material to such a region should induce the formation of a dorsal lip there if the cortex does possess these properties. The grafting experiments show that this type of grafting has exactly this effect, and provides evidence for the definite existence of morphogenetic properties in the cortex.

Grafting experiments allow investigation of the manner in which one part of the cortex interacts with another. Since the cortical structure remained intact under centrifugation or inversion it was impossible to do this heretofore. The results of grafting between various sites which have been described lead to limited conclusions, for only a few of the very many possible grafts have as yet been done. Moreover, it should be remembered that the results described apply to *Xenopus* alone—it is possible that different degrees of cortical interaction occur in other species.

Nevertheless, it is possible to draw some conclusions about the nature of the cortical field. The results suggest that no one portion of the cortex controls the remainder completely, for no graft is completely altered by, or completely alters, the surrounding cortex. Each graft retains, in respect of its ability to invaginate at gastrulation or not, the properties it would have shown in its normal position. Moreover, it is of interest to note that Schechtman (1942) found that grafts of cellular portions of the very early gastrula made in the same way behaved in a very similar manner in gastrulation. These autonomous characters shown by portions of the cortex in gastrulation may not extend to their other morphogenetic properties. At present there is no information on such matters as the regulation of size in organs formed from the graft or nearby tissues. Nor is it known what is the precise course of differentiation in the cells derived from the graft and around it. Dollander (1950) showed that a reorganization of the cortical field might occur after ligature of early stages of *Triturus helveticus*, which indicates a fair degree of interaction between parts of the cortex.

Attention should be drawn to several other matters. In these experiments no close check was kept on the size of graft: what would be the effect of larger or smaller grafts? In addition, the graft was inserted without regard to its orientation—presumably in the number of experiments done a very wide range of orientations were used. That the results of each type of grafting were very consistent suggests that orientation, at least in grafts of the size used, does not affect the display of the cortical properties. Some of the embryos wounded in the grey crescent region produced 'ring' embryos, as did some of those receiving grafts in this region. Schechtman (1942) was able to show in *Hyla regilla* that separation of the lateral marginal regions from the dorsal lip shortly before gastrulation, by the interposition of inert materials, produced such embryos. It is possible that the 'ring' embryos described here are due to such an effect, if it be supposed that grafting can sometimes damage the cortex in such a way that barriers to the interaction of dorsal lip and lateral marginal zones are formed.

The significance of the results of grafting infertile material to fertile embryos is at present obscure. They do, however, show that the cortex of fertile and unfertile eggs is different and that this difference can be transferred at least from infertile to fertile eggs. That the cortex is deeply involved in fertilization and cleavage is of course well known (see Waddington, 1956). The experiments of Runnström (1958) with sea-urchin eggs may be a close parallel to these results. He was able to prevent the cortical reaction from spreading to the whole egg on fertilization: in consequence cleavage was inhibited. It would be very interesting to know what happens to mitotic processes in such embryos. Closely related are the grafts between embryos of differing age which again show a transfer of properties with the graft and whose results relate to mechanisms of cleavage control.

SUMMARY

1. A technique for the grafting of cortical material from one embryo to another at early cleavage stages is described. This method may also be used for the grafting of cortical material from infertile eggs to early cleavage stages.

2. Grafts transplanted to embryos during first cleavage to the same site as that from which they were derived, resulted in normal development. Grafts obtained from grey crescent material and transplanted to the ventral margin induced the appearance of a secondary dorsal lip and subsequent secondary axis. Those of

animal pole or ventral margin material placed in the grey crescent resulted in the failure of invagination during gastrulation at the graft site, and this causes the splitting of the dorsal lip into two and the appearance of double axes. Animal pole material placed at the vegetal pole produces a peculiar form of exogastrulation.

3. Cortical material from infertile eggs if grafted into embryos during their first cleavage inhibits further development. Cortical material from fertile uncleaved eggs, if placed in embryos in the midst of their first cleavage, appears to cause a lag in cleavage in that half of the embryo which received the graft.

4. These results show that the cortical material definitely possesses morphogenetic properties which may be transferred with it. These are concerned with gastrulation, and the experiments are discussed in relation to the 'cortical field' theory of Dalcq & Pasteels. This technique of grafting allows investigation of the organization of the cortical field.

RÉSUMÉ

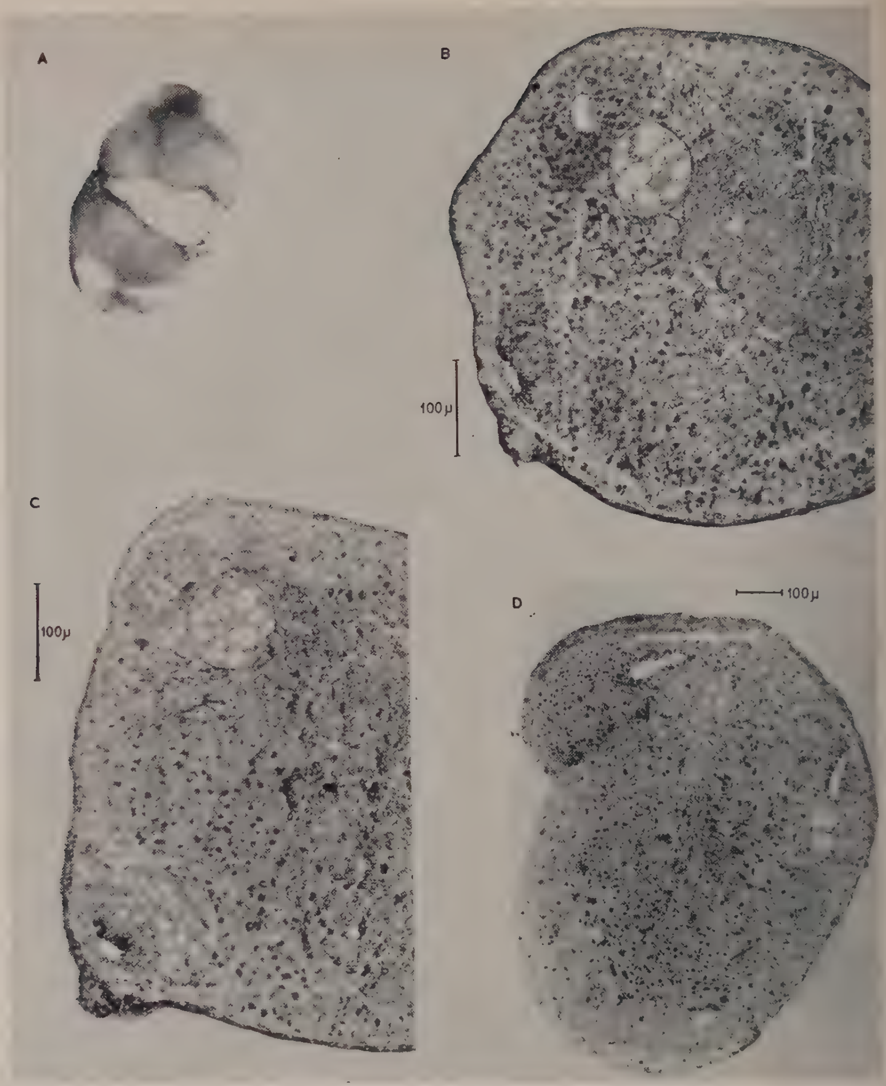
Transplantations du cortex chez Xenopus laevis

1. Une technique décrite ici permet de transplanter du matériel cortical d'un œuf à un autre aux stades initiaux de la segmentation. Cette méthode peut aussi être employée pour la transplantation de matériel cortical des œufs non fécondés aux œufs en début de clivage.

2. Les greffes reportées pendant la première segmentation au même endroit que celui dont elles provenaient ont été suivies d'un développement normal. Les transplantations de matériel du croissant gris à la région marginale ventrale ont causé l'apparition d'une lèvre blastoporale secondaire et subséquemment d'un système secondaire d'organes axiaux. Lorsque du matériel provenant du pôle animal ou de la zone marginale ventrale est placé dans le croissant gris, l'invagination gastruléenne fait défaut à cet endroit, d'où une séparation en deux de la lèvre dorsale avec formation de deux systèmes axiaux.

3. Les greffes à des œufs en première segmentation de matériel cortical provenant d'œufs vierges inhibent leur développement ultérieur. Lorsque du matériel cortical provenant d'œufs fécondés indivis est implanté dans des œufs en voie de leur première division, il produit un retard de la segmentation dans la moitié de l'œuf qui a reçu le greffon.

4. Ces résultats montrent que le matériel cortical possède effectivement des propriétés morphogénétiques qui peuvent être transposées avec lui. Elles concernent la gastrulation et les résultats sont discutés en relation avec la théorie du 'champ cortical' de Dalcq et Pasteels. Cette technique de transplantation rend possible l'investigation de l'organisation du champ cortical.



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EXPLANATION OF PLATE

FIG. A. Whole embryo of *Xenopus laevis* at neural plate stage. Secondary neural plate and dorsal lip induced by graft from grey crescent to ventral margin visible on upper end of embryo. ×15.

FIG. B. Transverse section through whole embryo at stage 26, which received same type of graft as illustrated in fig. A. Two neural tubes visible, one with notochord, the second anterior to notochord. Celestin blue and eosin.

FIG. C. Transverse section through same embryo as in fig. B, but more posterior. Two neural tubes and notochords visible.

FIG. D. Transverse section through embryo at stage 26 which received graft of animal pole material in grey crescent. Two closely contiguous neural tubes and notochords are to be seen. Celestin blue and eosin.

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Incorporation de l'adénine-C^{14} dans l'embryon de Poulet

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AVEC UNE PLANCHE

MALGRÉ le grand nombre de données déjà publiées sur le métabolisme des acides nucléiques pendant le développement embryonnaire, quelques aspects de cette question complexe méritent d'être reconsidérés, surtout quand on dispose de méthodes de recherche précises.

L'application de la méthode autoradiographique a permis déjà à quelques auteurs de suivre, au niveau tissulaire, l'incorporation de molécules marquées dans l'embryon de poulet: Smith & Gray (1948) avec le Cu^{64} et Hunt & Wolken (1948) avec le P^{32}, sur des embryons *in toto*; Feldman & Waddington (1955) et Pantelouris & Mulherkar (1957) avec la méthionine-S^{35}, sur des coupes histologiques. Tencer (1956), au moyen de cette même méthode, a suivi l'incorporation de l'adénine-C^{14} dans l'embryon de poulet aux stades de la ligne primitive, de 4 et de 9 somites; elle a surtout étudié la synthèse de l'acide désoxyribonucléique (DNA).

Nous avons repris la question en essayant de mettre en évidence l'incorporation du même précurseur dans d'autres constituants cellulaires d'embryons de poulet âgés de 1, 4, 5, 8, et 12 somites.

MATÉRIEL ET MÉTHODES

Les embryons ont été explantés aux stades compris entre le repli céphalique et 7 somites; leur développement a continué *in vitro* dans le 'minimum medium' de Spratt (1948), consistant en une solution saline physiologique tamponnée, additionnée de glucose et de rouge de phénol comme indicateur. Le précurseur, sulfate d'adénine-8-C^{14} semihydratée (Radiochemical Centre, Amersham, England), a été additionné directement au milieu de culture dans la proportion de 6–13 γ/c.c., de façon à obtenir une activité d'environ 0,3 μC./c.c. Les embryons ont continué leur développement dans ce milieu pendant une à trois heures,

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avant d'être fixés au Serra (éthanol 6: formol 3: acide acétique 1) durant une heure. Après enrobage à la paraffine selon la méthode usuelle, les coupes de 10 μ ont été étalées sur six lames, déparaffinées et traitées pendant une à deux heures dans une solution d'adénine non marquée (0,05 pour cent) afin de parachever l'élimination de l'adénine-C^{14} libre. Pour tous les embryons, des séries de coupes ont été soumises aux traitements suivants: (*a*) ribonucléase (Armour Laboratories) à 0,5 mg./c.c. pendant deux heures à 37° C.; (*b*) eau distillée dans les mêmes conditions; et (*c*) acide chlorhydrique normal pendant cinq minutes à 60° C. Après rinçage soigné et séchage à l'air, les lames ont été autoradiographiées, selon la technique de Ficq (1955*a*), avec de l'émulsion photographique pour recherches nucléaires G_5 Ilford 'in gel form'. Dans tous les cas, l'exposition a été de trois jours. Après développement, fixation et séchage, on a procédé à la coloration des coupes avec le mélange d'Unna-Pappenheim au vert de méthyle-pyronine, selon la méthode de Brachet (1942).

Pour calculer la radioactivité de chaque territoire embryonnaire, on a compté le nombre de traces dans une unité de surface de 441 μ^2. Pour toutes les lames, on a analysé le même nombre de coupes, tant périphériques que centrales. Le background cosmique a toujours été décompté.

RÉSULTATS

Toutes les données numériques ont été consignées dans les tableaux 1 à 5. Les chiffres expriment les activités enregistrées à trois niveaux pris sur l'axe céphalo-caudal: *A*, *B*, *C*; pour chacun de ces niveaux, on a analysé, dans le système nerveux, trois niveaux dans le sens dorso-ventral.

TABLEAU 1

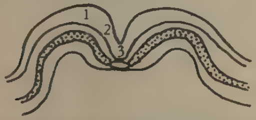
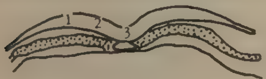
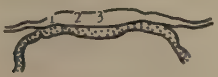
Stade: 1 somite Adénine: 13γ/ 0,3 μC./c.c./1 heure Nombre de traces/441 μ^2				*Incorporation totale*	*Acides nucléiques*	*Protéines*	*Fraction hydrosoluble à 37° C.*
A		Système nerveux	1	1,57	1,34	0,71	0
			2	2,88	1,25	0,72	0,91
			3	2,79	1,94	0,80	0,05
		Chorde		..	..	..	..
		Mésoblaste		..	..	..	..
B		Système nerveux	1	7,19	1,52	1,25	4,50
			2	5,37	1,81	0,72	2,84
			3	4,63	1,43	1,34	2,11
		Chorde		3,41	1,97	1,08	0,94
		Mésoblaste		5,80	2,45	0,62	2,73
C		Système nerveux	1	4,97	0,51	1,09	3,83
			2	7,20	0,48	1,36	5,89
			3	5,18	0	1,70	4,12
		Chorde		4,11	..	..	..
		Mésoblaste		4,70	..	0,70	3,03

TABLEAU 2

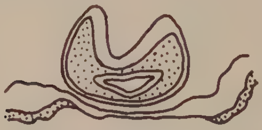
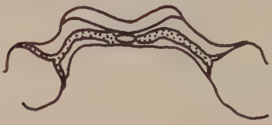
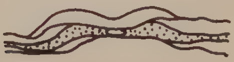
Stade: 4 somites Adénine: 13 γ/0,3 μC./c.c./1 heure Nombre de traces/441 μ^2				*Incorporation totale*	*Acides nucléiques*	*Protéines*	*Fraction hydrosoluble à 37° C.*
A		Système nerveux	1	1,43	0,76	0,12	0,55
			2	1,97	1,13	0,55	0,46
			3	3,41	1,51	0,96	0,94
		Chorde		. .	. .	. .	. .
		Mésoblaste		. .	. .	. .	. .
B		Système nerveux	1	2,65	2,53	0,12	0
			2	3,65	1,71	0,42	1,52
			3	5,88	3,06	0,40	2,42
		Chorde		4,61	2,30	0,29	2,02
		Mésoblaste		3,83	2,37	0,46	1,00
C		Système nerveux	1	5,36	4,08	0,71	0,57
			2	7,01	4,40	0,96	1,65
			3	7,95	4,99	1,79	1,17
		Chorde		3,83	3,76	0	0,07
		Mésoblaste		6,31	4,57	0,61	1,13

TABLEAU 3

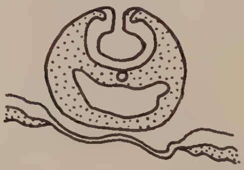
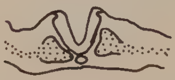
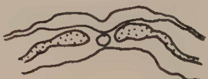
Stade: 5 somites Adénine: 13 γ/0,3 μC./c.c./1 heure Nombre de traces/441 μ^2				*Incorporation totale*	*Acides nucléiques*	*Protéines*	*Fraction hydrosoluble à 37° C.*
A		Système nerveux	1	0,34	0,12	0,22	0
			2	0,66	0,16	0,44	0,16
			3	1,04	0,47	0,08	0,60
		Chorde		0,95	. .	. .	. .
		Mésoblaste		. .	. .	. .	. .
B		Système nerveux	1	2,24	1,32	0,53	0,39
			2	3,22	1,90	0,59	0,73
			3	5,00	3,12	1,39	0,49
		Chorde		2,95	2,38	0,57	0
		Mésoblaste		4,12	2,97	1,15	0
C		Système nerveux	1	6,76	5,27	0	1,49
			2	6,36	6,36	0	0
			3	7,22	6,60	0	0,62
		Chorde		4,73	2,10	0	2,63
		Mésoblaste		6,43	4,98	0	1,45

TABLEAU 4

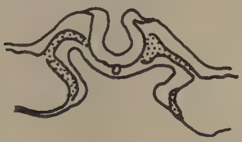
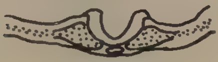
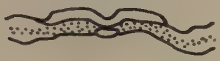
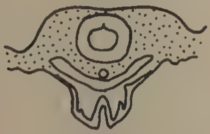
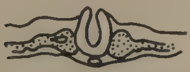
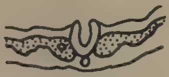
Stade: 5 somites Adenine: 6 γ/0,3 μC./c.c./3 heures Nombre de traces/441 μ^2			*Incorporation totale*	*Acides nucléiques*	*Protéines*	*Fraction hydrosoluble à 37° C.*
A 	Système nerveux	1	0,61	0,25	0,52	0
		2	2,17	0,55	0,52	1,10
		3	4,65	0,76	1,66	2,99
	Chorde		3,79	0,40	1,57	2,47
	Mésoblaste		..	..	..	..
B 	Système nerveux	1	8,16	2,40	1,53	4,23
		2	14,55	5,44	1,72	7,39
		3	23,91	9,58	2,41	11,92
	Chorde		11	7,08	1,24	2,68
	Mésoblaste		18,12	6,63	1,38	10,11
C 	Système nerveux	1	13,88	3,50	2,16	8,22
		2	15,31	4,92	1,85	8,54
		3	14,19	5,09	1,57	7,53
	Chorde		25,83	0,68	2,24	23,17
	Mésoblaste		23,42	5,97	2,64	14,81

TABLEAU 5

Stade: 8 somites Adénine: 6 γ/0,3 μC./c.c./2 heures Nombre de traces/441 μ^2			*Incorporation totale*	*Acides nucléiques*	*Protéines*	*Fraction hydrosoluble à 37° C.*
A 	Système nerveux	1	2,07	0,12	1,33	1,16
		2	1,56	0,33	1,87	0,03
		3	1,68	0,90	0,96	0
	Chorde		0,93	..	1,53	0
	Mésoblaste		..	..	..	..
B 	Système nerveux	1	1,97	0,22	0,96	1,14
		2	3,00	0,77	0,98	1,37
		3	6,64	4,25	0,58	1,81
	Chorde		3,93	2,29	0,20	1,44
	Mésoblaste		5,71	2,10	2,04	2,43
C 	Système nerveux	1	3,31	1,00	5,03	0
		2	7,50	2,38	4,28	0,95
		3	12,59	4,52	5,64	2,43
	Chorde		8,09	4,95	4,20	0
	Mésoblaste		10,27	7,08	6,77	0,04

Dans le système nerveux, l'adénine-C^{14} s'est incorporée en suivant des gradients bien définis dans tous les embryons, exception faite du plus jeune, âgé d'un somite, où la distribution s'est montrée moins régulière. L'un des gradients décroît dans le sens caudo-céphalique de l'axe embryonnaire. Dans un embryon de 12 somites coupé frontalement, on a pu suivre exactement ce gradient décroissant de la queue vers la tête avec une remarquable régularité (Fig. 1). Un second gradient décroît dans le sens ventro-dorsal, sur toute la longueur du système nerveux. Ce gradient se montre moins marqué à l'extrémité caudale de l'axe nerveux (niveau C; Planche).

Il est possible que dans la chorde dorsale il y ait aussi un gradient caudo-céphalique; mais sa faible activité et la surface de section réduite de la chorde ne permettent pas de tirer des conclusions avec certitude. Son activité peut dépasser celle des crêtes neurales; mais elle est, en général, inférieure à celle de la paroi ventrale de la gouttière neurale (niveau 3).

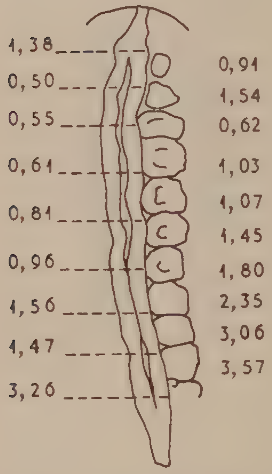


FIG. 1. Activités correspondantes au système nerveux (à gauche), et au mésoblaste paraxial (à droite) sur une coupe frontale.

Dans le cas du mésoblaste paraxial, nous n'avons tenu compte que des somites; dans le mésenchyme céphalique, la densité cellulaire était trop basse pour fournir des résultats valables. Dans les somites, on peut retrouver le gradient caudo-céphalique (Fig. 1). Leur activité dépasse, en général, celle de la chorde; mais elle reste inférieure à celle de la région ventrale du système nerveux.

Nous avons essayé d'étudier l'incorporation de l'adénine marquée dans l'acide ribonucléique, l'acide désoxyribonucléique et les protéines, au moyen de l'hydrolyse par la ribonucléase et l'acide chlorhydrique normal; mais les chiffres obtenus montrent un degré de dispersion trop élevé, peut-être dû au peu d'activité restante après hydrolyse, pour qu'on puisse tirer des conclusions de ces données.

L'eau seule à 37° C. enlève d'ailleurs une activité déjà assez importante, mais sans affecter beaucoup la coloration par la pyronine. Il s'agit peut-être, d'une fraction du précurseur liée à des nucléosides ou des nucléotides, dont on connaît la grande solubilité dans l'eau chaude.

Dans notre matériel, l'adénine montre une incorporation préférentielle dans les acides nucléiques et dans cette fraction soluble dans l'eau tiède. Elle semble, cependant, s'incorporer aussi dans des protéines, après avoir sans doute subî une dégradation, comme on l'a déjà trouvé dans les oocytes de Batraciens (Ficq, 1955*b*). Cette incorporation dans les protéines devient plus importante dans l'embryon plus âgé (8 somites).

L'adénine incorporée dans les acides nucléiques montre une distribution selon les deux gradients déjà décrits: caudo-céphalique et ventro-dorsal; en ce qui concerne les fractions protéique et soluble dans l'eau, il est plus difficile de l'affirmer.

DISCUSSION

Puisque nous avons traité les embryons de poulet avec des échantillons d'adénine-C^{14} d'activités diverses et pendant des laps de temps différents (1 à 2 heures), nous nous abstiendrons de donner une valeur absolue à nos chiffres. Nous nous bornerons à considérer la distribution relative de la radioactivité pour chaque embryon étudié.

Nos résultats confirment qu'il se produit une incorporation d'adénine dans les acides nucléiques de l'embryon de poulet pendant son développement initial, comme Tencer (1956) l'avait déjà constaté par la méthode autoradiographique. Nous avons pu confirmer aussi que l'incorporation est plus importante dans le neurectoblaste que dans le mésoblaste. Mais nous avons trouvé que le précurseur suit une distribution différente dans l'axe nerveux de celle décrite par Tencer, qui a trouvé un gradient décroissant dans le sens céphalo-caudal aux stades de ligne primitive et de 4 somites, et caudo-céphalique au stade de 9 somites; Tencer a décrit aussi un second gradient dorso-ventral, avec un maximum dans les crêtes neurales. Nous avons trouvé aussi une répartition suivant ces deux directions, mais en sens *inverse*: caudo-céphalique et ventro-dorsal. Le gradient ventro-dorsal est surtout marqué dans les régions moyennes du tronc, où la gouttière neurale est en train de former le tube neural. Cette constatation nous fait croire que les acides nucléiques doivent jouer un rôle dans ce processus morphogénétique.

Nous croyons qu'il existe un rapport intéressant entre nos données et celles trouvées par Lavarack (1957) sur la topochimie de l'acide ribonucléique dans l'embryon de poulet. Cet auteur a trouvé que, pendant la transformation de la plaque en tube neural, le cytoplasme des cellules en contact avec la paroi profonde de la gouttière neurale montre une remarquable richesse en RNA. Sa coloration par la pyronine présente un aspect beaucoup plus homogène que dans les régions plus superficielles de l'epithélium neural. L'auteur a mis en évidence le fait significatif que c'est précisément cette face du système nerveux, très colorable à la pyronine, qui se trouve en contact avec le chordomésoblaste. Cette concentration basale de RNA présente son maximum à l'extrémité céphalique, pour s'effacer à l'extrémité caudale. Malgré la présence de l'émulsion photographique sur nos coupes, nous avons pu retrouver ces images, qui disparaissent après traitement à la ribonucléase ou à l'acide chlorhydrique. Mais nous n'avons pas pu constater de gradient d'intensité céphalo-caudal. S'il est vrai que le contact avec le chordomésoblaste active le métabolisme du RNA neural, il devient aisé de trouver une signification aux gradients ventro-dorsaux que nous avons observé dans la distribution de l'adénine: la région ventrale de la gouttière neurale incorporerait plus de précurseur sous l'influence du chordomésoblaste que les régions plus dorsales, qui échapperaient à son influence.

Le fait que, dans l'embryon, la distribution du RNA coincide avec celle des protéines sulfhydrilées (Brachet, 1947) justifie la comparaison de nos résultats avec ceux de Feldman & Waddington (1955), qui ont étudié l'incorporation de la méthionine-S^{35}, cet acide aminé essentiel qui s'incorpore dans les protéines soufrées. La distribution de ce précurseur dans l'embryon de poulet concorde en deux points avec celle que nous avons trouvée pour l'adénine: (*a*) concentration maximum dans le centre nerveux; (*b*) concentration plus importante dans les somites que dans la chorde. Mais elle diffère dans le cas des gradients formés dans le tube neural: la méthionine-S^{35} suit un gradient céphalo-caudal et dorso-ventral qui, concordant avec ceux décrits par Tencer (1956), sont inverses de ceux que nous avons trouvé pour l'adénine-C^{14}. Pantelouris & Mulherkar (1957), en employant aussi la méthionine-S^{35}, mais sur des embryons cultivés *in vitro*, affirment avoir trouvé une distribution de l'isotope similaire à celle décrite par Feldman & Waddington.

RÉSUMÉ

1. L'incorporation d'adénine-C^{14} a été étudiée dans des embryons de poulet se développant *in vitro* dans le 'milieu minimum' de Spratt. La localisation a été décelée par autoradiographie des coupes 'histologiques'.

2. Dans les embryons à 4, 5, 8 et 12 somites, l'adénine est surtout incorporée dans le tube neural et cela suivant deux gradients: caudo-céphalique et ventro-dorsal.

3. Les somites montrent une incorporation plus réduite et la chorde dorsale encore moindre.

4. La plus grande partie de l'adénine paraît être incorporée dans les acides nucléiques et dans une fraction soluble dans l'eau qui est présumée être formée de nucléotides.

SUMMARY

The incorporation of adenine-C^{14} *into chick embryos*

1. The incorporation of adenine-C^{14} into chick embryos developing *in vitro*, in Spratt's 'minimum medium', has been studied. Its localization has been detected by employing autoradiography on histological sections.

2. In embryos of 4, 5, 8, and 12 somites, adenine is mainly incorporated into the neural tube according to two gradients: caudo-cephalic and ventro-dorsal.

3. Somites showed a lower incorporation, and the notochord even less.

4. The greater part of the adenine appears to be incorporated in nucleic acids and in a water-soluble fraction which we presume is composed of nucleotides.

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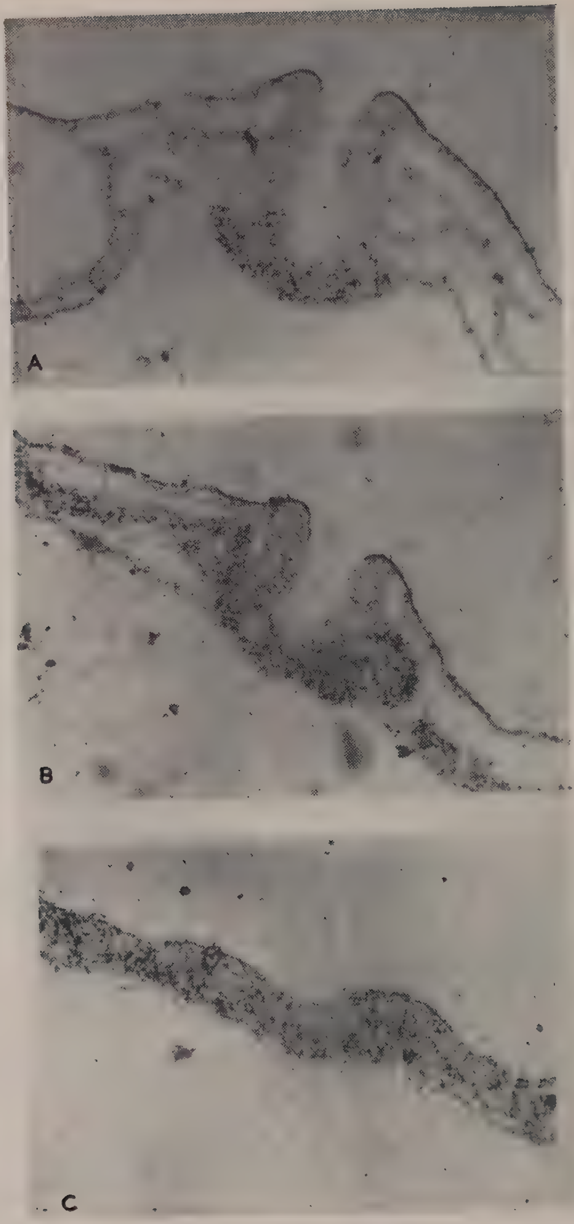

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EXPLICATION DE LA PLANCHE

FIG. A. Embryon de 5 somites (adénine: 6 γ/0,3 μC./c.c./3 h.): Niveau *A*.
FIG. B. Idem: Niveau *B*.
FIG. C. Idem: Niveau *C*.

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The Functional Differentiation of the Small Intestine

VII. The Duodenum of the Foetal Guinea-Pig, with a Note on the Growth of the Adrenals

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WITH TWO PLATES

INTRODUCTION

THE epithelium of the duodenal mucosa of vertebrates is strikingly uniform. In all but the cyclostomes and a few fishes, the epithelium is made up of tall columnar absorbing cells, with mucus-producing goblet cells interspersed. The columnar cells have a high cytoplasmic/nuclear ratio, with the cytoplasm arranged into an apical zone containing a prominent Golgi apparatus, and a basal zone. The apical surface is featured by a sheet of microvilli that are extremely rich in alkaline phosphatase. The attainment of these characteristics has been shown to take place in a brief critical period, just before hatching in the chick embryo (Moog, 1950) and just before birth in the mouse (Moog, 1951), although in the latter animal a second period of phosphatase accumulation precedes weaning. The guinea-pig has now been added to this comparative series, because the protracted gestation period (about 67 days) might permit some further insight into the problems of duodenal differentiation. The newborn guinea-pig is quite mature and is at once able to eat solid food. According to the extensive work of Flexner and his colleagues (cf. Flexner, 1955), the cerebral cortex of the foetal guinea-pig reaches a high degree of maturity a full month before birth. The lungs also attain an advanced state of differentiation during prenatal life (Sorokin, Padykula, & Herman, 1959). In the duodenal epithelium slight phosphatase activity is detectable histochemically by the end of the 7th week (Verne & Hébert, 1949). The striated border is present by that time, but other aspects of chemical and cytological differentiation are said to be delayed until the 2nd or 3rd day after birth (Peyrot, 1955). This delay in differentiation appears to be related to the fact that the epithelial cells, during most of the foetal period, are choked with glycogen (Peyrot, 1957). The present study

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suggests that the foetal epithelium is a storage site for glycogen, which is used at the time of birth.

In both the chick embryo and nursling mouse, normal differentiation of the duodenal epithelium is dependent on the secretions of the adrenal cortex (Moog, 1953, 1959*a*; Moog & Richardson, 1955). Whether this is also true for the foetal guinea-pig is not known. It is, however, shown in this paper that the adrenal glands achieve their maximum relative weight at 32 days *in utero*, and that the cortex is rich in sudanophilic substances (suggesting the possibility of secretion) throughout the period of duodenal differentiation. Pennacchietti (1938), on the basis of cytological observations, has also advanced the view that the adrenal cortex of the foetal guinea-pig is functional throughout the second half of intra-uterine life.

MATERIALS AND METHODS

A total of 158 foetal and infant guinea-pigs (59 litters) was used in this investigation. These animals were from a colony maintained in the Zoology Department of the University of Chicago. Gestation age was dated from the morning after observed copulation. At the appropriate time, the pregnant female was killed by decapitation and was bled, the abdomen was opened, and the foetuses were removed as rapidly as possible. Each foetus was weighed and its sex was recorded. About 1 cm. of the foetal duodenum posterior to the entrance of the common bile duct was removed, weighed on a torsion balance, and placed in either iced distilled water or a fixative. The foetal adrenals were also dissected out and weighed, and in some cases fixed.

Alkaline phosphatase activity was determined in total homogenates at a tissue concentration of 2–5 mg. per ml. Phenylphosphate was generally used as substrate, but in some cases, to be described later, beta-glycerophosphate was used. In either case, substrate concentration was 50 mM, the pH was 9·6 at 37° C. (carbonate-veronal buffer), and $MgCl_2$ was added at a final concentration of 10 mM. These conditions were established as optimal by preliminary tests on tissue from foetuses 45 and 60 days old. In contrast to the situation in the chick embryo and mouse, where optimal conditions vary according to substrate and stage of development (Moog, 1959*b*), the same optima obtained in the guinea-pig at both stages and with both substrates tested. When phenylphosphate was employed, liberated phenol was measured colorimetrically with the Folin–Ciocalteu reagent; when beta-glycerophosphate was used, liberated phosphate was measured by the Fiske–Subbarow (1925) technique. Total nitrogen content was determined by direct Nesslerization after digestion of an aliquot of the homogenate.

For histochemical observation of the duodenum, fixation was usually carried out in 85 per cent. alcohol at —5° C. The tissue was embedded in paraffin and sectioned at 7 μ. From each duodenal sample some sections were stained with Delafield's haematoxylin and erythrosin, and others were treated according to the following techniques: (1) staining of basophilic substances in 0·1 per cent. toluidine blue (Moog & Wenger, 1952), with and without prior exposure to 10 per cent. perchloric acid at 4° C. to extract ribonucleic acid (Erickson, Sax, & Ogur, 1949); (2) staining of polysaccharides by the periodic acid Schiff (PAS) technique (McManus, 1948), with and without prior digestion in 0·1 per cent. malt diastase to remove glycogen (Lillie, Greco, & Laskey, 1949); (3) staining of sites of alkaline phosphatase activity, with beta-glycerophosphate as substrate (Gomori, 1952).

In a few cases the adrenal glands were fixed for 1 to 3 weeks in formol-cobalt for demonstration of sudanophilic substances according to McManus's method as described by Pearse (1954). These glands were embedded in paraffin, sectioned at 7 μ, and mounted serially.

RESULTS

Quantitative measurement of phosphatase activity and nitrogen accumulation

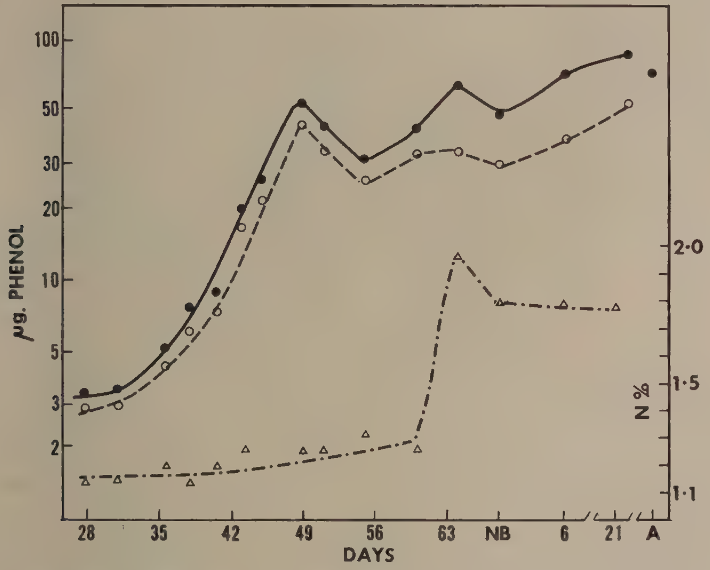
Total nitrogen concentration

Between 28 and 60 days *in utero*, the percentage of nitrogen in the duodenum rises very slightly (Text-fig. 1). Between 60 and 64 days, however, the nitrogen concentration undergoes a swift and significant increase to 1·96 per cent. (Table 1). This high value was maintained in a single litter still unborn at 68 days, but it had fallen to 1·77 per cent. in the new-born animals (i.e. within 18 hours of birth). During the first 3 weeks after birth the nitrogen concentration does not change significantly.

Alkaline phosphatase activity

At 28 days, the earliest stage used for enzyme determination, alkaline phosphatase activity is at the low level characteristic of tissues in which there is no functional accumulation of this enzyme (Table 1). By 36 days a definite increase is detected, and thereafter the values climb steadily to a peak at 49 days (Text-fig. 1). There is then a decrease of 46 per cent. to a lower value at 55 days, followed by a small but still significant increase to 60 days (Table 1). When activities are expressed in terms of wet weight there is an apparent rise again, but this becomes negligible when the values are expressed in terms of nitrogen content. Activity drops very slightly at birth, but during the first 3 weeks rises to almost twice the birth level. The adult activity, which was obtained only on a wet weight basis, is not much different from that of the 6-day-old infant. The single litter still unborn at 68 days gave anomalously low values. It is interesting that all values obtained in the guinea-pig duodenum are very much lower than those previously obtained for that of the hatched chick or of the weanling mouse (i.e. new-born guinea-pig, about 3 μg. phenol liberated per μg. N in $\frac{1}{2}$ hour; 1-day-old chick, 100 μg. phenol; 18-day-old mouse, 300 μg. phenol). The three animals do, however, strikingly resemble each other in that the periods of intensive phosphatase accumulation in all cases culminate in a transient maximum which is followed by a prompt decline; thus the 49-day peak in the guinea-pig duodenum (Text-fig. 1) has its counterpart in a peak achieved just after hatching in the chick, and at birth and again at 18 days in the mouse.

In the chick embryo (Kato, 1960) and young mouse (Moog, 1959*b*), the normal increase of phosphatase activity cannot be attributed to appearance of a dissociable activator, or to loss of a dissociable inhibitor. In the foetal guinea-pig this point was checked by combining equal parts of homogenates obtained from 35- and 55-day embryos. The activity of the former was 2·4 μg. phenol liberated per mg. fresh weight per half hour, that of the latter 46·2 μg.; the combined homogenates liberated 25·7 μg., which is close to the numerical average of the two activities.



TEXT-FIG. 1. Accumulation of alkaline phosphatase and nitrogen in the duodenum of the developing guinea-pig. The ordinate on the left refers to the amount of phenol liberated in 30 minutes. The upper graph (solid line and circles) represents phosphatase activity per mg. fresh weight; the graph just below (dash line and open circles) represents activity per 10 μg. total nitrogen. The lower graph (dot-dash line and triangles) shows total nitrogen as per cent. of fresh weight.

Weight and sex in relation to phosphatase activity

As was previously found in the chick and mouse, phosphatase activities in the developing duodenum are quite variable (Table 1). Sizeable differences were sometimes found among members of a single litter. The observed differences in phosphatase activities at any given age could not be correlated with sex, body-weight, litter size (which influences body-weight after the end of the 7th week), or absolute or relative adrenal weight.

TABLE 1

Phosphatase (PH) *and nitrogen in the duodenum of the foetal guinea-pig*

PH is expressed as μg. phenol liberated per half hour, nitrogen as per cent. of fresh weight

Age (days)	*Number*		*% Nitrogen* (mean±s.e.)‡*	PH/*mg. wet weight (mean and range)*	PH/*10 μg.* N *(mean±s.e.)*	*P*†
	Foetuses	*Litters*				
28	5	2	1·12±0·08	3·4 (2·5–4·2)	3·1±0·38	
31	8	3	1·14±0·06	3·6 (2·0–6·8)	3·2±0·65	
36	16	6	1·19±0·03	5·4 (2·4–11·5)	4·5±0·67	<0·005
38	7	2	1·12±0·03	7·8 (4·5–10·8)	6·9±0·92	
40	3	1	1·20±0·10	8·7 (8·0–9·0)	7·2±0·27	<0·005
43	5	2	1·19±0·09	21·6 (11·2–39·0)	18·1±14·5	
45	32	12	1·25±0·04	26·1 (8·5–46·7)	20·8±1·96	<0·001
49	5	2	1·24±0·05	56·3 (46·7–72·0)	45·3±3·38	
52	3	1	1·24±0·07	43·9 (32·6–56·8)	36·6±5·64	<0·005
55	11	4	1·30±0·06	31·9 (17·0–50·5)	25·6±1·67	
60	25	9	1·25±0·01	45·0 (15·5–65·0)	33·8±2·75	<0·01
64	6	2	1·96±0·07	67·9 (31·5–97·7)	34·6±5·0	
68 (*in utero*)	3	1	1·98	22·7 (16·8–29·2)	11·4	
New-born	17	7	1·77±0·07	52·7 (17·5–96·2)	29·8±3·3	
6 (*post-natal*)	7	3	1·78±0·08	70·6 (35·2–154)	40·2±8·8	<0·02
21	5	3	1·72±0·05	96·4 (67·0–165)	56·0±6·7	
Adult§	3	3		69·1 (47·3–80·0)		

* The increase in per cent. total nitrogen between 60 and 64 days, and the decrease between 64 days and birth, are highly significant ($P < 0·001$ for both).

† Probabilities refer to the difference between the mean values at the ends of the brackets.

‡ Standard error of the mean.

§ Pregnant females.

TABLE 2

Relative rates of hydrolysis of phenylphosphate (PhP) *and beta-glycerophosphate* (bGlP) *by homogenates of foetal guinea-pig duodenum at different stages of development*

Activity is given as μg. phosphorus liberated per mg. fresh tissue in 30 minutes. With PhP, activity has been converted to amount of phosphorus equivalent to the amount of liberated phenol actually measured

Age (days)	*Number of samples*	*Activity*		*Ratio* PhP/bGlP *(mean±s.e.)**	*Probability*
		With PhP *(mean and range)*	*With* bGlP		
31	2	1·3 (1·24–1·36)	1·9 (1·88–1·92)	0·68	
45	13	11·1 (3·6–15·8)	9·6 (2·2–14·3)	1.22±0·072	$P < 0·001$
60	13	15·2 (6·8–21·7)	18·5 (5·8–27·4)	0·81±0·044	

* Standard error of the mean.

Rates of hydrolysis of phenylphosphate and beta-glycerophosphate

Purified phosphatase from the duodenal mucosa of the calf hydrolyses phenylphosphate about 2·4 times as fast as beta-glycerophosphate (Morton, 1955). Kato (1960) has observed that with the chick embryo duodenum the ratio of P liberated from phenylphosphate to P liberated from beta-glycerophosphate (PhP / bGlP) is not constant, but rises from 1·8 at 14 days to 4·3 at 17 days, and then falls to 2·4 at hatching. A similar rise during the period of phosphatase increase has been demonstrated in the young mouse (Moog, 1959*b*). In the foetal guinea-pig this ratio has been determined at only 3 stages (Table 2). These data do, however, indicate that in this animal too the activity against phenylphosphate is at a peak during the period of rapid increase of phosphatase activity.

Histochemical observations on the developing duodenum

Since the histological and histochemical observations that have been made in this study are, with minor exceptions, in agreement with those of Peyrot (1955, 1957), they will be considered here only briefly.

General structure of the duodenal mucosa

At 26 days the lumen is largely occluded by a mass of vacuolated epithelial cells with large nuclei; only narrow discontinuous passageways penetrate the mass (Plate 1, fig. A). At 35 days the villi are demarcated at their bases and sides, where they are covered by a single layer of large epithelial cells in which the nuclei are irregularly arranged. In our material the villi tips continue to be joined together by masses of relatively small epithelial cells, so that the lumen comprises a meshwork of openings as late as the middle of the 6th week (Plate 1, fig. B; Plate 2, fig. E). Peyrot did not observe this blocking of the lumen beyond the 27th day.

By 49 days the villi are free at their distal ends, although masses of apparently extruded cells are still found in the lumen. The epithelial cells are still large and vacuolated, but their arrangement is more regular than before, with the nuclei rather uniformly situated in the basal halves of the cells. Not until 64 days, however, does the epithelium begin to assume the appearance of the mature functional condition.

Basophilia

Up to 64 days the epithelial cells at the sides and tips of the villi show very little cytoplasmic basophilia after staining with toluidine blue; by 43 days there is, however, some basophilia, extractable with perchloric acid in the cold, at the villi bases and in the apparently extruded cells. At 64 days and subsequently, the cytoplasm assumes a granular appearance, and stains well with toluidine blue. Since the ability to take up this stain is also removable with perchloric acid, it is probably due to the presence of RNA. During most of the period under examination, the nuclei in the crypts are small and densely stained, but those on the outer halves of the villi are larger and paler, so that numerous fine granules may be seen within them. No metachromatic reactions appeared anywhere in the sections at any stage.

Polysaccharides

As Peyrot (1957) has already shown, the duodenal epithelium is heavily charged with glycogen throughout the foetal period. In the present study, large granules of glycogen (i.e. PAS-positive material extractable with diastase) were scattered abundantly in the epithelial mass in both alcohol- and Bouin-fixed material at 26 days (Plate 1, fig. A). At 35 days and later, glycogen aggregates as large as 2 or 3 cells were distributed throughout the epithelium; in many cases the entire cytoplasmic area seemed to be filled with the stained material (Plate 1, fig. C). These observations support Peyrot's (1957) contention that the vacuolated appearance of cells stained by other techniques is due to the presence of very large quantities of glycogen. The cells are still choked with the substance as late as the 67th day, but just after birth this has disappeared (Plate 1, fig. D).

Digesting sections with diastase prior to application of the PAS technique makes it possible to see the polysaccharides that are not digestible. Rather surprisingly, a thin but fairly well-defined striated border is present already at 35 days. This has a somewhat shaggy appearance at first (Plate 1, fig. B), as Peyrot (1955) has also observed, but becomes thick and smooth at 49 days. A small number of mucus-filled goblet cells are present at 49 days, and these become steadily more numerous (Plate 1, fig. D). Paneth cells are occasionally seen at 43 days, are numerous at 49 days, and become very conspicuous by 55 days; as in the adult guinea-pig (Zaccheo & Viale, 1957), these cells contain glycogen and abundant polysaccharide of a non-digestible type (Plate 1, fig. D).

Localization of phosphatase

At 26 days the only sign of phosphatase activity is a slight, questionable reaction on the surfaces of the epithelial nuclei after 2 hours of incubation. At 35 days small reactive spots can sometimes be seen on the surfaces of the uncompleted villi after 15 minutes of incubation. But even after 60 minutes, the surface reaction remains slight and patchy, whereas the subepithelial region of the mucosa is uniformly stained, indicating that much of the activity determined quantitatively at this stage is due to mucosal, and not to epithelial, phosphatase (Plate 2, fig. E). At 43 days a rather irregular reaction is obtained in the striated border on the villi sides and bases after only 2 minutes, but the reaction is still irregular after 60 minutes; the mucosa now stains very lightly. At 49 days a distinct and continuous border reaction extending from the tips of the villi to their bases appears after 2 minutes of incubation (Plate 2, fig. F), and the same reaction is then obtained in all subsequent stages.

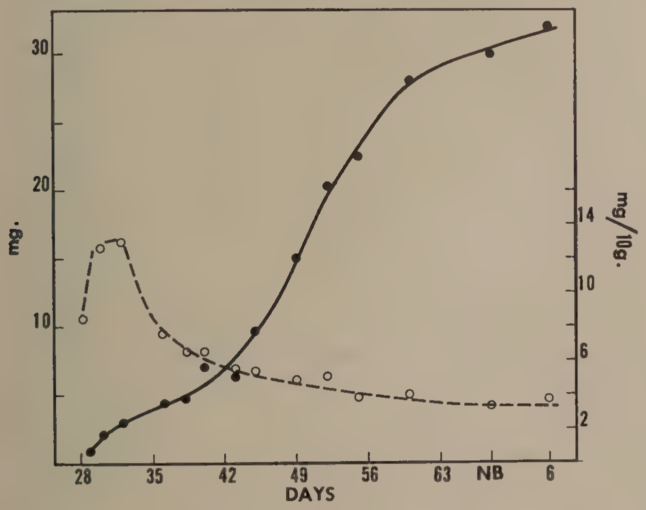
An interesting peculiarity of the foetal guinea-pig's epithelium is that no phosphatase reaction appears in the apical cytoplasm at later stages, even after

60 minutes of incubation (cf. figs. F and G, Plate 2). Ordinarily the staining of the apical cytoplasm is ascribed to 'diffusion' of the reaction product from the highly active border (see, for example, Johansen & Linderstrom-Lang, 1952), although the enzyme has been shown to be present after removal of the border (Fredriccson, 1956). The failure of enzyme or reaction product to 'diffuse' in the present case militates against the general interpretation that an apical reaction is due to spreading from a site of high activity. The lack of spreading might, however, be due to the relatively low phosphatase activity in the guinea-pig's duodenum (see p. 184).

The adrenal glands

Growth

In females, the adrenals grow steadily from the earliest stage examined (29 days) until birth (Text-fig. 2); in males, growth stops in the 8th week, although a transient increase occurs between 60 and 64 days (Table 3). Between 29 and 32 days there is a significant increase in adrenal weight per unit of body-weight, at least in females, followed by a highly significant decrease in both sexes (Table 3). After 43 days the adrenal weight per unit of body-weight is fairly uniform. Relative weight does not differ between the two sexes at 32 or 36 days, but at 45 days a difference appears that is thereafter sustained. (The failure to demonstrate a significant sex difference at 55 days is probably due to a sampling



Text-fig. 2. The growth of the adrenal glands in the developing guinea-pig. The solid line represents increase in absolute weight per pair of adrenals (ordinate on the left). The dash line represents adrenal weight per 10 g. body-weight (ordinate on the right).

error.) In relative terms, the weight shifts in males between 60 and 64 days and birth are not significant (Table 3).

These results show that the adrenal weight per unit of body-weight is at a maximum at the time when duodenal phosphatase begins its rise (compare Text-figs. 1, 2). Examination of serial sections of 35-, 43-, and 50-day adrenals reveals that the cortex is large and indeed extends through the medulla throughout this period (Plate 2, fig. H). Thus it appears that the body is richly furnished with cortical material early in the period of phosphatase increase in the duodenum.

TABLE 3

The growth of the adrenal glands in the foetal guinea-pig

Age (days)	*Number*		*Actual weight (mg./pair)*		*Relative weight* (mg./pair/10 g. body-weight)*		*P*†
	♀	♂	♀	♂	♀	♂	
29	3	1	0·97	1·70	8·42±1·72‡	13·6	
30	1		2·20		12·72		
32	5	7	3·24	3·13	13·01±1·21	13·72±1·12	>0·5
36	4	5	4·55	4·56	8·99±0·47	8·86±0·41	>0·5
38	3	3	4·85	5·74	6·59	7·39	
40	3		7·21		6·74		
43	3	2	6·39	4·26	4·95	3·96	
45	8	7	9·80	9·13	5·44±0·19	4·48±0·32	<0·025
49	2	3	15·1	13·9	4·99	3·65	
52	1	2	20·4	21·5	5·20	4·44	
55	8	4	22·5	20·3	3·96±0·27	3·63±0·35	>0·4
60	8	4	27·9	21·6	4·05±0·14	3·23±0·13	<0·005
64		5		31·0		3·72±0·30	
New-born	8	5	30·1	21·2	3·46±0·18	2·54±0·28	<0·02
6	3	3	32·0	30·8	3·75	3·54	

* The significance of changes in relative weight has been tested. For females, increase in relative weight from 29 to 32 days, $P < 0{\cdot}05$; decrease from 32 to 36 days, $P < 0{\cdot}01$. For males, decrease from 32 to 36 days, $P < 0{\cdot}005$; decrease from 64 days to birth, $P > 0{\cdot}4$.

† These values refer to the relative weight difference between males and females on the day indicated.

‡ Standard error of the mean. These values have been calculated for series in which there are three or more individuals of the same sex from two or more litters.

Sudanophilia

Already at 35 days the adrenal cortex is organized into a zona glomerulosa and a zona fasciculata in which the columnar arrangement of cells is beginning to appear. The cells of the fasciculate zone are very large and stain well with Sudan black at all three stages examined (Plate 2, fig. H), although the intensity of staining seemed to increase from 35 to 50 days. The strands of cortical cells criss-crossing the medulla are also sudanophilic, though less so than the more peripheral area. On the basis of cell size and apparent lipid content, it seems reasonable to assume that the adrenal cortex has the capacity to function by the 36th day.

DISCUSSION

The studies reported here demonstrate that the alkaline phosphatase of the foetal guinea-pig duodenum rises to the adult level during a critical period. This period is not as abrupt and sharply delimited as those previously demonstrated in the more rapidly developing chick embryo and mouse (Moog, 1950, 1951). Nevertheless, enzyme activity does clearly rise, between 35 and 49 days, from the low level characteristic of phosphatase-poor tissues to the adult level. Other observations also suggest that the 49th day marks the culmination of an important period of development in the duodenum. The lumen is first completely open during the 7th week, an event probably correlated with the onset of swallowing at 45 days (Becker, Windle, Barth, & Schulz, 1940). The striated border, though sketched in at the end of the 5th week, seems well differentiated by the end of the 7th. The first mucus-filled goblet cells are seen at 49 days, and the first Paneth cells, with their characteristic chemistry, at 43 days.

These events in the development of the duodenum coincide strikingly in time with critical changes in the development of the cerebral cortex and the lungs. The extensive studies of Flexner and his co-workers (cf. Flexner, 1955) on the differentiation of the cerebral cortex have established the period from about 41 to 45 days as the time of functional maturation. During this period the neurones attain the morphology characteristic of the functional state; they accumulate Nissl substance (Peters & Flexner, 1950), their nuclei mature (La Velle, 1956), their permeability appears to be altered (Flexner, 1955), and they undergo some important enzymatic changes (Flexner & Flexner, 1948; Flexner, Belknap, & Flexner, 1958). Recently, Sorokin, Padykula, & Herman (1959) have defined the 47th day as the point of attainment of an advanced state of chemo-architectural differentiation in the lungs. The fact that three quite different organs should undergo intensive differentiation simultaneously suggests that they may all be controlled by one extrinsic factor. It may be significant that relative adrenal weight is at a maximum shortly before the onset of critical changes in brain, lungs, and intestine.

The period of intensive change in the duodenal epithelium of the guinea-pig differs in some important respects from similar periods in the chick embryo and foetal mouse. In both these latter animals a series of morphological and chemical changes occur in rapid sequence (Moog, 1950, 1951); in the chick embryo, at least, these events cannot be dissociated by hormonal stimulation. In the foetal guinea-pig, on the other hand, the sequence is normally dissociated. A few examples will illustrate this point. The accumulation of phosphatase in all three animals is correlated with the construction of a polysaccharide-rich border. Yet in the guinea-pig the border does not form as part of a general morphogenetic pattern in which the epithelial cells are fully differentiated to the functional level, but appears on the surfaces of vacuolated cells which, as Peyrot (1957) has already shown, are little more than storage sacs for glycogen. The development of the typical elongate epithelial cell with its dense apical and basal masses of cytoplasm is delayed almost to the time of birth. This late differentiation is probably reflected in the delayed period of nitrogen accumulation (Text-fig. 1). In both chick embryo and foetal mouse the increase in nitrogen concentration largely parallels the increase in phosphatase.

The patterns of functional differentiation in the chick embryo and mouse cannot, however, be entirely opposed to that in the foetal guinea-pig. The most significant finding of the second study in this series (Moog, 1951) was that, in the mouse, the accumulation of phosphatase is split into two periods which are more than 2 weeks apart. The mucus cells also vary in time of differentiation among the three animals, appearing during the single critical period in the chick embryo (Moog & Richardson, 1955), after birth in the mouse (Peyrot, 1955), and after the time of phosphatase accumulation in the guinea-pig foetus. The Paneth cells show a similar difference between guinea-pig and mouse (Peyrot, 1955).

The patterns of glycogen accumulation in the duodenal epithelium pose a problem of special interest. In the chick embryo glycogen accumulates to a peak at 18 days and then declines rapidly, as other aspects of functional differentiation are accelerated (Moog & Thomas, 1955). As far as one can judge from histochemical evidence, the pattern in the foetal mouse is similar (Peyrot, 1957). But in the guinea-pig the duodenal epithelium is choked with glycogen throughout the foetal period. This glycogen might possibly be a reservoir of carbohydrates for use in the construction of the striated border, mucus cells, and other polysaccharide-rich structures, as Peyrot has suggested; but this seems unlikely because the differentiation of the epithelium is accompanied by little or no visible diminution of the glycogen. It seems, rather, that the foetal duodenal epithelium of the guinea-pig, not yet having assumed its proper function, is exploited as a storage site for carbohydrate that will sustain the young animal through the transition to extra-uterine life.

The chemical and structural uniformity of the mature intestinal epithelium among vertebrates has already been remarked (see introduction). The facts just discussed make it clear that what is held in common among different vertebrates is the end result only. The steps by which that end result is achieved are plainly adaptable to the needs of the developmental history of the species.

This divergence between end and means may cast some light on the nature of evolutionary change. The uniformity of the end result bespeaks a genetic substratum that has persisted unaltered throughout the course of vertebrate evolution. The diversity of the means, on the other hand, shows that the common genetic mechanism does not operate uniformly, the time of action of the relevant genes being apparently shifted along the time axis of development. Seen from this point of view, the non-dissociability of the differentiative events in the chick embryo duodenum becomes merely incidental, and it is not surprising to learn that it is possible to dissociate the accumulation of phosphatase from other aspects of differentiation; the administration of a phosphatase-inducer to the developing system has such an effect (Kato, 1960). The transition from one developmental type to another in the course of evolution might be attributed to some change resulting in the precocious activation of one gene, or the prolonging of inhibition of another. The effective factor might be a modifying gene, or it might be a cytoplasmic difference traceable to an antecedent stage, perhaps even to the ovum. Since it is now generally believed that there is continuous interaction between nucleus and cytoplasm or environment (cf. Stern, 1955; Markert, 1958), a small change occurring at a strategic moment might result in a considerable alteration of the overall pattern.

The only extrinsic factor thus far clearly implicated in the differentiation of the small intestine is the secretion of the adrenal cortex. In the chick embryo (Moog & Richardson, 1955) and young mouse (Moog, 1953), adrenocortical secretion seems to be essential to the realization of the normal patterns of differentiation, and the evidence available suggests that this may also be true for the guinea-pig during the 6th and 7th weeks of intra-uterine life. But it is not possible to attribute the differences in patterns of duodenal development to differences in adrenocortical activity. In the chick embryo we may reasonably attribute glycogen accumulation and loss, nitrogen accumulation, phosphatase increase, and goblet-cell differentiation, to the presence of an enhanced quantity of corticoid in the blood-stream (cf. Moog, 1959*a*); but the same events in the foetal guinea-pig cannot be due to a single upsurge of cortical activity, since they are separated from each other in time. Granted that the adrenal cortex has an essential role to play, the regulation of metabolic patterns and their chemo-architectural consequences must ultimately rest in the genetic apparatus of the reacting cells (cf. Moog, 1959*b*). Hormones are effective only when a condition of responsiveness exists. A striking example of change in responsiveness of what must be a gene-controlled reaction has recently been produced by Nemeth & Nachmias (1958), who have shown that substrate administration does not elicit tryptophane peroxidase activity in the foetal guinea-pig liver until the stage of development at which the enzyme normally increases.

SUMMARY

1. The development of the duodenal epithelium of the foetal guinea-pig from the 29th day *in utero* to 3 weeks after birth has been studied by histochemical and quantitative biochemical techniques. The growth of the adrenal glands has also been studied.

2. Alkaline phosphatase accumulates in the striated border of the epithelial cells as the definitive villi are cut out of a mass of epithelium that occludes the intestinal lumen at early stages. The villi are completed early in the 7th week, and phosphatase activity, measured against phenylphosphate, reaches its maximal value at 49 days.

3. The activity against beta-glycerophosphate does not rise in parallel with

that against phenylphosphate. The ratio of amount of phosphorus liberated from phenylphosphate to amount liberated from beta-glycerophosphate is significantly higher at 45 days than at 31 or 60 days.

4. At the time of alkaline phosphatase accumulation and later, the epithelial cells are choked with glycogen and appear vacuolated when stained by any technique that does not demonstrate glycogen. During the last week *in utero* glycogen seems to be used up, and it is not found in the new-born duodenum.

5. Total nitrogen concentration does not increase until after 60 days *in utero*. In the same period granular basophilic cytoplasm begins to replace the vacuoles predominant in earlier stages.

6. The results suggest that the end of the 6th and the beginning of the 7th prenatal weeks constitutes a critical period for the functional differentiation of the foetal duodenal epithelium. Other authors have shown that the same period is critical for the differentiation of the cerebral cortex and the lungs.

7. The adrenal glands achieve their maximal relative weight at the middle of the 5th week. The cortex is large at this time, and is rich in sudanophilic substances. It appears capable of secreting during the critical period described above. A difference in relative weight of male and female adrenals appears first at 45 days.

8. Comparison of patterns of duodenal differentiation in the guinea-pig, chick embryo, and mouse indicates that the chemical and structural similarity of mature intestinal epithelia is arrived at by different developmental pathways.

RÉSUMÉ

La différenciation fonctionnelle de l'intestin grêle

VII. Le duodénum fœtal du cobaye, avec une note sur la croissance des surrénales

1. Le développement de l'épithélium duodénal du cobaye a été étudié du 29^e^ jour de la vie utérine à la 3^e^ semaine après la naissance à la fois par des méthodes histo-chimiques et des dosages biochimiques. La croissance des glandes surrénales a également été étudiée.

2. La phosphatase alcaline s'accumule dans le plateau strié des cellules épithéliales lorsque les villosités définitives se dégagent de la masse épithéliale qui comble aux jeunes stades la lumière de l'intestin. Les villosités sont complètement formées au début de la 7ème semaine, et l'activité phosphatasique, mesurée en employant le phényl-phosphate, atteint sa valeur maximal à 49 jours.

3. L'activité en présence de béta-glycérophosphate ne s'élève pas parallèlement à celle du phénylphosphate. La proportion entre la quantité de phosphate libéré du phénylphosphate et celle libérée du béta-glycérophosphate est significativement plus haute à 45 qu'à 60 jours.

4. Au moment où la phosphatase alcaline s'accumule et plus tard, les cellules épithéliales sont gorgées de glycogène et apparaissent vacuolisées si elles sont colorées par toute technique qui ne démontre pas le glycogène. Pendant la dernière semaine de la vie utérine, le glycogène paraît être utilisé, et ne se trouve plus dans le duodénum du nouveau-né.

5. La concentration totale des composés azotés n'augmente pas jusqu'après le 60^e jour de la vie utérine. A la même période, le cytoplasme granulaire basophile commence à remplacer les vacuoles prédominant aux plus jeunes stades.

6. Les résultats suggèrent que la fin de la 6^e semaine prénatale et le début de la 7^e représentent une période critique pour la différenciation fonctionnelle du duodénum fœtal. D'autres auteurs ont montré que la même période est également critique pour la différenciation du cortex cérébral et des poumons.

7. Les glandes surrénales atteignent leur poids relatif maximum au milieu de la 5^e semaine. A ce moment leur cortex est considérable et riche en substances soudanophiles. Il paraît capable d'activité sécrétoire pendant la période critique signalée ci-dessus. Une différence entre les poids relatifs des surrénales mâles et femelles n'apparaît qu'au 45^e jour.

8. La comparaison des types de différenciation surrénale chez le cobaye, l'embryon de poulet et la souris indique que la similitude structurale et chimique de l'épithélium intestinal à sa maturité est atteinte par un développement qui suit des voies différentes.

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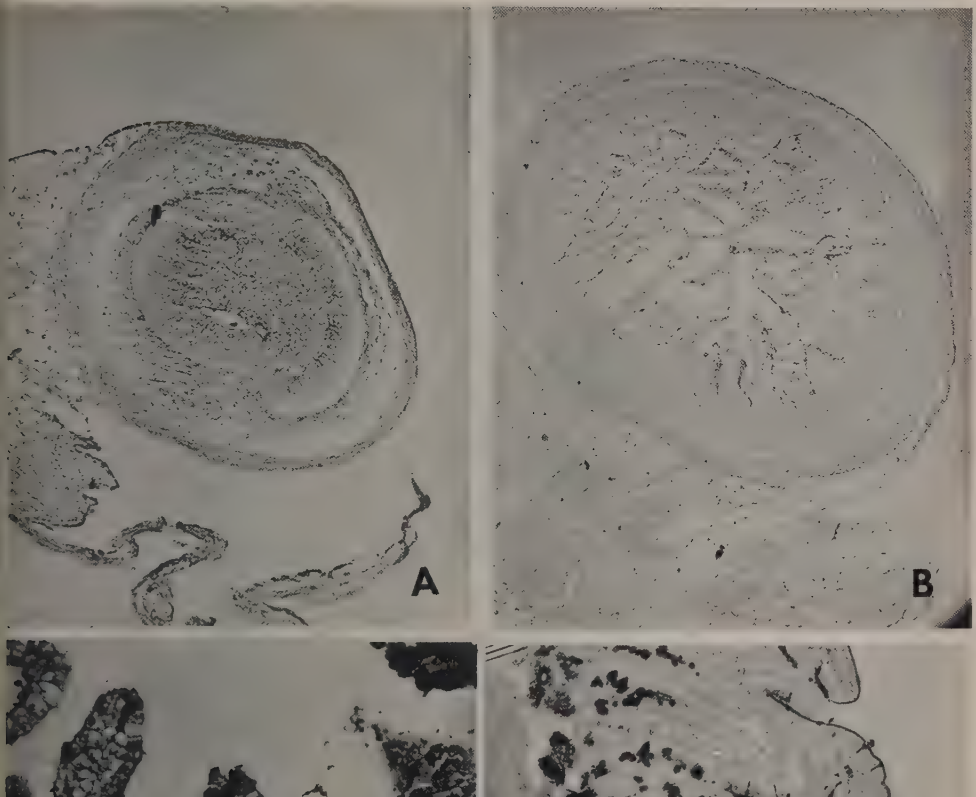

F. MOOG *and* E. ORTIZ

Plate 1

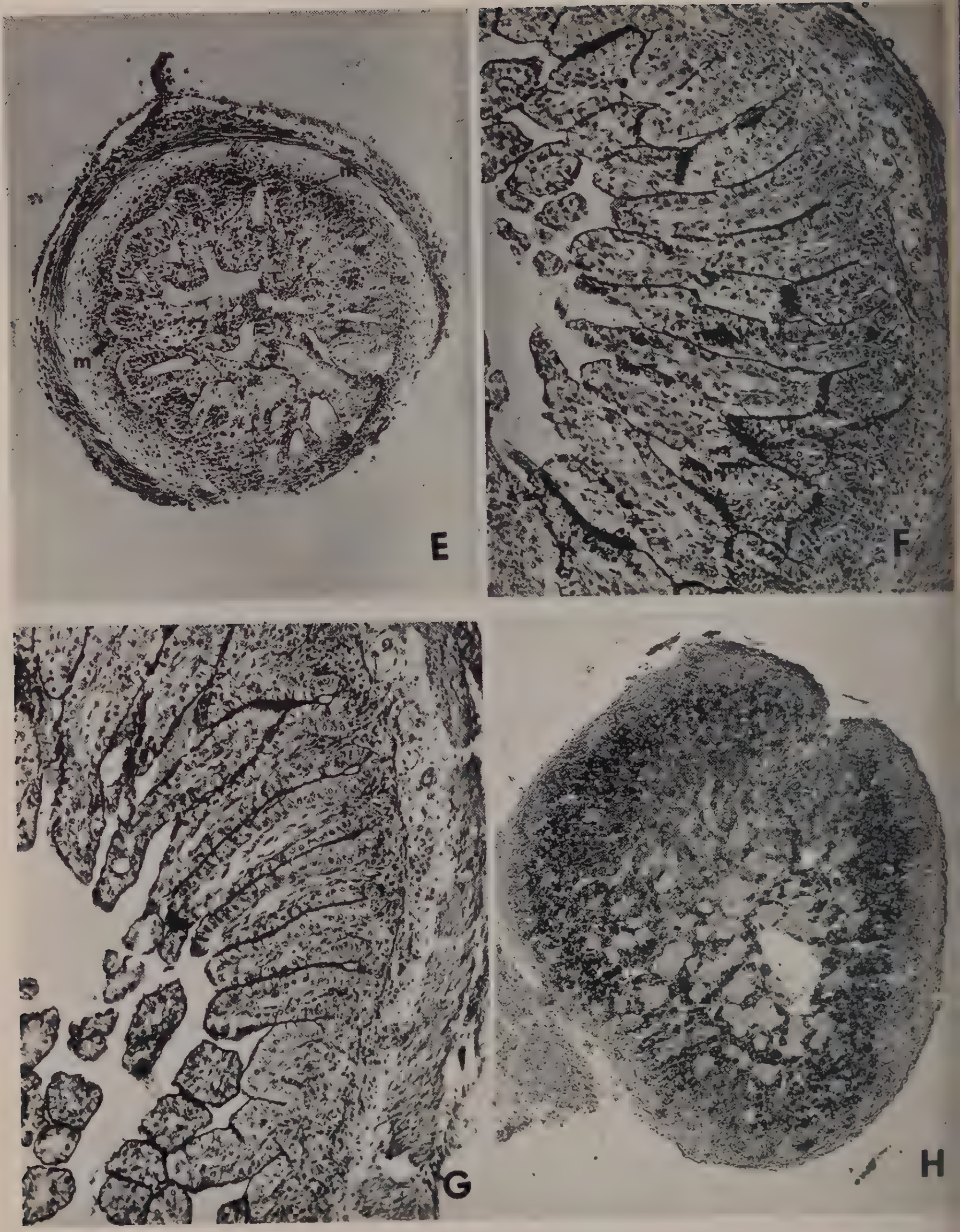

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Plate 2

mucosa del canale alimentare di animali sottoposti a rialimentati. *Biol. Lat. Milano*, **10,** 576–601.

EXPLANATION OF PLATES

PLATE 1

Foetal guinea-pig duodenum sectioned at 7 μ and stained by the periodic acid/Schiff technique to reveal polysaccharides. The section shown in fig. B was treated with diastase to remove glycogen.

FIG. A. 26 days. The lumen is occluded by a mass of epithelial cells containing glycogen (fine black dots). × 85.

FIG. B. 43 days. The lumen is still blocked by a meshwork of processes, on the surfaces of which shaggy PAS-positive borders may be seen. Glycogen has been removed. × 80.

FIG. C. 64 days. The epithelial cells appear to be completely filled with glycogen. × 225.

FIG. D. New-born. No glycogen is present. Polysaccharides are present in the striated border, which is now well developed; in the goblet cells (*g*), which are most abundant in the crypts; and in the Paneth cells (*p*) below the crypts. × 100.

PLATE 2

FIGS. E–G are of foetal guinea-pig duodenum sectioned at 7 μ and treated according to the Gomori technique for alkaline phosphatase; the counterstain is erythrosin.

FIG. E. 38 days. 1-hour incubation. An uneven reaction may be seen on the surfaces of the villous processes. There is a marked reaction in the sub-epithelial mucosa (*m*). Darkening of other areas is not due to phosphatase activity. × 70.

FIG. F. 49 days. 2-minute incubation. Only the striated borders of the villous epithelial cells show a reaction. × 155.

FIG. G. 49 days. 60-minute incubation. Reactivity is still confined to the striated borders. Note that there has been no diffusion into the cytoplasm of the epithelial cells, despite the prolonged incubation (cp. fig. F). × 155.

FIG. H. Adrenal gland of 50-day guinea-pig foetus fixed in McManus cobalt-formol and stained with Sudan black B. The stain has been taken up by the zona fasciculata and its extensions into the medulla. The zona glomerulosa remains unstained. Particularly on the right, the columnar arrangement of the fasciculate cells is discernible. × 80.

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Studies on Embryonic Determination of the Harlequin-fly, *Chironomus dorsalis*

I. Effects of centrifugation and of its Combination with Constriction and Puncturing

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WITH TWO PLATES

INTRODUCTION

THERE have been several published works on dipteran embryonic determination (Reith, 1925; Pauli, 1927; Howland *et al.*, 1935, 1936, 1941; Counce & Selman, 1955). These papers, however, deal primarily with the development of higher Diptera such as the house-fly and the fruit-fly. Furthermore, the eggs of such higher Dipterans are surrounded by tough and opaque chorions which often hinder detailed observations.

The eggs of the harlequin-fly, on the contrary, are known to be very transparent, and as a matter of fact these eggs were extensively used during the first quarter of this century for the tracing of the germ line.

In the present experiment the eggs of *Chironomus dorsalis* were used for two purposes: (1) to observe directly changes within the egg after various treatments, taking advantage of their transparency, and (2) to contribute to the analysis of the developmental mechanism of lower members of the Diptera.

MATERIAL AND METHODS

The egg

Eggs of *C. dorsalis* were collected in the field. Since the eggs are enclosed in a jelly mass the jelly was dissolved by sodium-hypochlorite when necessary.

The polarity of the eggs can be discerned by the general shape of the egg and by the position of the micropyle; that is, the anterior side of the egg is flatter than the posterior side, and the micropyle is present in a small disk-like area which is located at the centre of the anterior side. Judgement of the polarity becomes easier after the appearance of the pole cells which are budded off into the 'posterior polar pocket' during the 4- to 8-cell stage.

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Centrifugation

Centrifugation with various orientations of the egg was chiefly employed, sometimes combined with constriction and puncturing of the egg.

To orient an egg with reference to the centrifugal axis it was first sucked into a fine glass tube snugly fitting the shorter axis of the egg, and then both ends of the tube were sealed with paraffin. In order to centrifuge along the long axis of the egg the glass tubing was simply inserted into an ordinary centrifuge tube and spun. To obtain an oblique stratification the glass tubing was fitted into a groove, running at the desired angle, which was cut out of a wooden board shaped to fit into the metal cup of the centrifuge. For centrifuging the eggs along their shorter axes no precaution was necessary since the eggs tended to lie on their flat sides.

For the simultaneous designation of the direction of the centrifugation and that of the egg axis, Howland's terminology (1941) will be adopted. According to her system, when the anterior end of the egg is centrifugal it is called anterior centrifugation, and the reverse is posterior centrifugation. In the case of oblique centrifugation there may be two kinds: anterior-lateral and posterior-lateral centrifugation.

The centrifugation time was kept constant at 10 minutes; the centrifugal force applied ranged between 100 g. and 30,000 g. After centrifugation the eggs were freed from the glass tubing and cultured in tap-water.

Constriction and puncturing

Constriction was performed by a simple tool made after Brauer & Taylor's design (1936).

Puncturing of the egg was done by a fine glass needle. Immediately after puncturing the egg was dipped into 30 per cent. ethanol for 30 seconds to make it form a cytoplasmic coagulum in order to seal off the wound and prevent further out-flowing of the egg contents.

EXPERIMENTAL RESULTS

(1) *Stratification of egg constituents*

Living, uncentrifuged eggs at early stages have a layer of transparent cytoplasm on the surface and the interior of the eggs is filled with refractile oil droplets and brown 'yolk' granules.

In sections, besides the superficial cytoplasmic layer, many cytoplasmic islands are found in the interior of the egg, each containing a nucleus. These cytoplasmic islands are connected with each other and also with the superficial layer by means of cytoplasmic strands.

Living eggs subjected to centrifugation of 3,000 g. are stratified into three zones, as shown in Plate 1, fig. A. When they are centrifuged along the long axis of the egg heavy dark-brown yolk granules are accumulated into the centrifugal two-fifths of the egg; grey oil drops are stratified into the centripetal fifth; between the two is a clear cytoplasmic zone. Although the nuclei are shifted to the centripetal side of the clear cytoplasmic zone, they quickly spread out into the entire clear zone immediately after centrifuging is stopped.

In sections of centrifuged eggs it is no longer possible to recognize either the superficial layer or the islands and strands, and the cytoplasm is simply massed into one zone which corresponds to the clear cytoplasmic zone of the living material.

In spite of the disturbance in the distribution of the contents, the pole cells are budded off at the posterior end of the egg at the usual place. Once the pole cells are formed their position is scarcely shifted by centrifugation.

The stratification of the constituents of the centrifuged *Chironomus* egg conforms qualitatively to that of the house-fly (Pauli, 1927) and of the fruit-fly (Howland, 1941), but it differs from them quantitatively. Since the eggs of both these latter species have only small amounts of inclusions, the cytoplasmic zone extends almost the entire length of the long axis of the egg.

It may be well to point out in passing that since the hyaline cytoplasm accumulates a little below the equator in the centripetal half of the egg of *Chironomus* (one-fifth to three-fifths of the long axis as measured from the light pole), the use of Howland's terminology sometimes becomes rather confusing. When the egg is centrifuged in the upright position, i.e. in anterior centrifugation, the cytoplasm is in the posterior half.

(2) *Redistribution after stratification*

The mode of redistribution of the stratified contents of the eggs differs according to the stage at which centrifugation is done and sometimes depends also upon whether centrifugation has been weak or strong.

In the stages before nuclear migration, with weak centrifugation (up to 150 g.), the strata begin to mix with each other as soon as the centrifuge is stopped.

After strong centrifugation (above 200 g.), although the strata look almost the same as those obtained by weak centrifugation, the zonation is always retained undisturbed until the nuclear migration stage. Furthermore, when redistribution begins, it occurs in such a way that the cytoplasm, together with the nuclei, spreads along the egg surface towards the two poles (Plate 1, fig. B, 1–4). By the time the tips of the returning cytoplasmic streams reach the poles almost all the cytoplasm is on the egg surface, and the yolk and oil zones, which remain unmixed, are passively pushed inward. In all of 50 eggs centrifuged strongly during the movement of the cytoplasm, the nuclei continue to divide and eventually a blastoderm which was, in appearance, no different from a normal one, was formed.

In eggs centrifuged in the syncytial blastoderm stage, redistribution begins immediately following the termination of centrifugation in both weakly and strongly centrifuged eggs, dispersion occurring along the surface in a pattern similar to that of the previous stage.

(3) *Centrifuging before nuclear migration and the resulting malformations*

Among the eggs strongly centrifuged before the nuclear migration stage various malformation types having a definite relation to the direction of centrifugation were obtained.

(*a*) *Posterior centrifugation*

From 750 eggs in which the transparent cytoplasm was stratified into the anterior half of the egg by posterior centrifugation, in 336 cases (9 / 150 by 200 g.; 14 / 150 by 760 g.; 43 / 150 by 1,700 g.; 113 / 150 by 3,000 g.; 138 / 150 by 25,000 g.) a constant abnormality was obtained which consisted of duplication of the head and absence of the thorax and abdomen (Plate 1, fig. C). Each half comprises the portion of head distal to the first maxilla, so that the two heads, connected by their respective first maxillary segments, are facing in opposite directions. Therefore, although one of the heads is directed in the normal direction, the other one is directed backward as if it has replaced the thorax and abdomen. This is a rather surprising result, since centrifuged eggs gave rise to a seemingly normal blastoderm after redistribution (see section 2). This type of abnormality will be referred to as ‘double cephalon’ in later paragraphs.

(*b*) *Anterior centrifugation*

On the other hand, another kind of double monster, consisting of duplicate sets of abdominal segments, was produced in 365 out of 750 centrifuged eggs (13 / 150 by 200 g.; 17 / 150 by 760 g.; 62 / 150 by 1,700 g.; 132 / 150 by 3,000 g.; 141 / 150 by 25,000 g.) after anterior centrifugation in which the cytoplasm is shifted into the posterior half of the egg. The two abdomens are connected by their respective second abdominal segments, pointing in opposite directions. The head and thorax are lacking altogether (Plate 1, fig. D). This monster will be called ‘double abdomen’.

(*c*) *Oblique centrifugation*

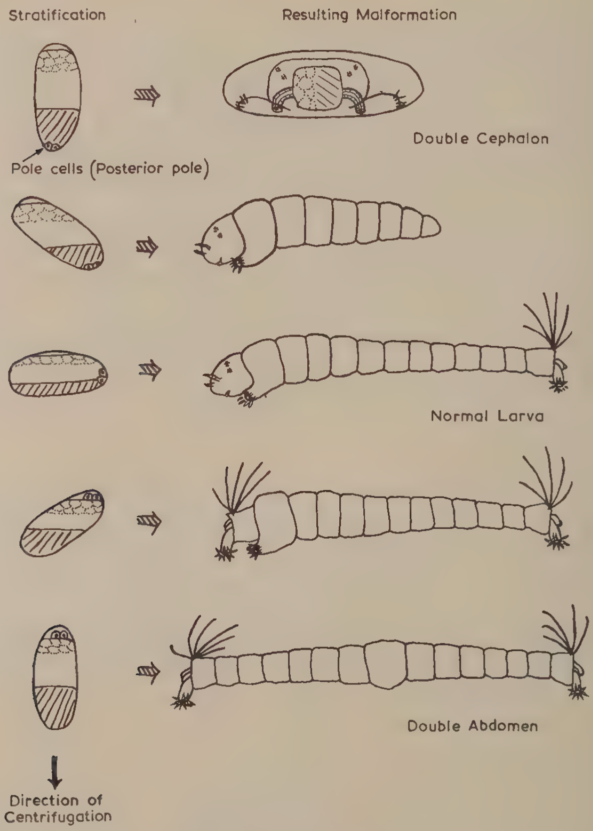
In the experiments with oblique centrifugation, only 3,000 g. was used. Malformation occurred in 132 of 150 centrifuged eggs.

(i) *Posterior-lateral centrifugation.* From the eggs subjected to posterior-lateral centrifugation, another type of double monster can be obtained. In this monster, a major part consisting of the segments from the cephalic end to the middle of the abdomen extends in the normal direction, and a minor part with only a few distal head segments runs in the reverse direction (Plate 1, figs. E, G).

(ii) *Anterior-lateral centrifugation.* The reverse of the situation found in (i), eggs subjected to anterior-lateral centrifugation produce monsters in which a major part consisting of the segments from the caudal end up to the middle of the thorax or head, follows the normal direction, while a minor part with only a few distal abdominal segments is attached to it in the reverse direction (Plate 1, figs. F, H).

(*d*) *Lateral centrifugation*

Normal larvae result from all the laterally centrifuged eggs in which the cytoplasmic zone extends along the entire long axis of the egg (Plate 1, fig. I).



TEXT-FIG. 1. Semi-diagrammatic drawings showing relationships between direction of centrifugation and type of resulting malformation.

In view of the fact that vertical centrifugation (anterior as well as posterior centrifugation) gives rise to double monsters, both members of which consist of equal numbers of segments which are mirror images of each other, and also of the fact that lateral centrifugation gives rise to normal embryos, it is extremely interesting that in oblique centrifugations, as the long axis of the egg is tilted further from the centrifugal axis, the number of segments belonging to the naturally directed portions becomes larger at the expense of the reversely directed portion (Text-fig. 1, Table 1).

TABLE 1

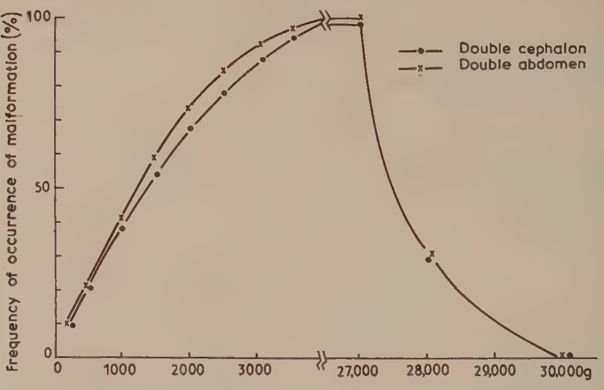
Relationships between degree of tilt of long axis of egg from the direction of centrifugation and composition of resulting malformation

	Degree of tilt of long axis from direction of centrifugation (°)	*Composition of resulting malformation*
Posterior centrifugation	0–10	Head segments before 1st maxilla+head segments before 1st maxilla in reverse direction
	30	Head+thorax+1st–3rd abdominal segments+few distal head segments in reverse direction
Posterior-lateral centrifugation	45	Head+thorax+1st–6th abdominal segments+few distal head segments in reverse direction
	60	Head+thorax+1st–8th abdominal segments+few distal head segments in reverse direction.
Lateral centrifugation	80–90	(Normal larva)
	60	9th abdominal segment in reverse direction+head segments behind antennal segment+thorax+abdomen
Anterior-lateral centrifugation	45	9th abdominal segment in reverse direction+thorax+abdomen
	30	6th–8th abdominal segments in reverse direction+metathorax+abdomen
Anterior centrifugation	0–10	2nd–9th abdominal segments in reverse direction+2nd–9th abdominal segments

Recently, Price (1958) reported the finding of a ‘unique monster embryo’ of the pitcher plant mosquito (Culicidae, Diptera). This monster is very similar to our ‘double-abdomen’, except that Price’s monster has two complete abdomens. Since the same kind of monster was also obtained in several other species of Chironomidae (the author, unpublished), the occurrence of double monsters may be common among the lower Diptera.

(4) *Frequency of occurrence of the malformations*

The frequency of occurrence of the malformations increases with increase in the centrifugal force. As Text-fig. 2 shows, the malformations are not produced by centrifugation below 200 g. At 200 g., one-tenth of all treated eggs develop into malformations. The value reaches about 100 per cent. at 3,500 g., and remains so up to about 27,000 g. At about 30,000 g., all eggs are killed during centrifugation.



TEXT-FIG. 2. Frequency of occurrence of malformations. Centrifugation time set at ten minutes.

(5) *Reduction of blastodermal size and modification of double malformation*

The structure of the double malformation is modified by decreasing the size of the blastoderm.

The heavy yolk or the light oil can be separated from the main part of the egg by centrifugation and thus the blastodermal size can be reduced. By using 20,000 g., the separation was obtained during the nuclear division stage (17 / 100 eggs), while by using 3,000 g. separation only occurred shortly before the nuclear migration stage (13 / 100 eggs). A similar type of embryo resulted from constricting with a thread unseparated eggs which had been centrifuged by 3,000 g. at early nuclear division stage.

The small blastoderm embryo also results from removing a part of the cytoplasm by puncturing the clear zone of the centrifuged egg. In this case the greater the volume of cytoplasm removed, the smaller the blastoderm becomes.

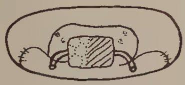
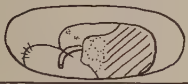
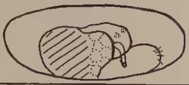
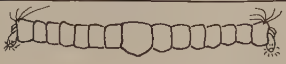
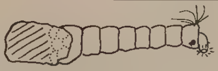
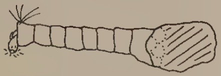
By decreasing the size of the blastoderm, the middle parts of the double monsters are lost to various extents and the number of missing segments increases in proportion to the decrease in the blastodermal size. Ordinarily the double cephalon has two heads consisting of 3 cerebral, the mandibular, and the first maxillary segments. But when the middle parts are lost with the reduction of the blastoderm, the omission proceeds in order from the maxilla to the mandible. In the reduced double abdomen, the omission spreads from the second abdominal segment towards the tail end.

These facts, together with the finding after oblique centrifugation that the reversely directed minor part is always terminal in structure, suggest that the terminal characters have a priority in development over the intermediate structures.

(6) *Effects of two successive centrifugations in the reverse directions*

As already stated in (2) and (3), a causal relationship can be found between the conditions of stratification and the resulting malformation types. The next problem is to test when these relationships are established. For this purpose, two successive centrifugations were applied to the same egg in reverse directions.

When the two centrifugations in reverse directions are given during the nuclear division stage, the effect of the earlier centrifugation is completely annulled and the resulting malformation type conforms to the second treatment.

	DEVELOPMENTAL STAGE AT CENTRIFUGATION			RESULTING MALFORMATION TYPE
	NUCLEAR DIVISION STAGE		SYNCYTIAL BLASTODERM STAGE	
COMBINATION OF SUCCESSIVE CENTRIFUGATIONS	ANTERIOR– CENTRIFUGATION	POSTERIOR CENTRIFUGATION		
	POSTERIOR CENTRIFUGATION		POSTERIOR CENTRIFUGATION	
	POSTERIOR CENTRIFUGATION		ANTERIOR CENTRIFUGATION	
	POSTERIOR CENTRIFUGATION	ANTERIOR CENTRIFUGATION		
	ANTERIOR CENTRIFUGATION		ANTERIOR CENTRIFUGATION	
	ANTERIOR CENTRIFUGATION		POSTERIOR CENTRIFUGATION	

TEXT-FIG. 3. Effects of two successive centrifugations in reverse directions upon type of malformation.

But if the later centrifugation is postponed until after the nuclear migration stage is over, i.e. until the syncytial blastoderm stage, although the gross nature of the embryos obtained (head or abdomen) is that expected from the *earlier* treatment, the doubleness is lost, the result being a partial or 'half' embryo closed at the truncated end (see Text-fig. 3). Here the *later* centrifugation determines which part of the double structure will persist. Expressing this more concretely, let us suppose that the first centrifugation is anterior and the second, given after the nuclear migration stage, is posterior centrifugation. From the first centrifugation a double abdomen is expected. After the second centrifugation, an abdomen is obtained with its caudal end pointing towards the anterior pole of the egg and its truncated end facing the posterior pole of the egg. It is obvious that this abdomen is the anterior member of a double abdomen which is selected because the cytoplasm is shifted to the anterior half of the egg by the second treatment.

The successive centrifugation experiment again shows that the gross developmental tendency towards head or abdomen is laid down during the nuclear migration stage and the second centrifugation can at most modify it to only a slight degree.

(7) *Centrifugation in advanced stages and the resulting malformations*

As stated in the preceding paragraph, the cephalic and abdominal characters are determined at about the nuclear migration stage. In the present section the changes of malformation type with advancing stage will be described.

During the stage from soon after nuclear migration to shortly before cellular blastoderm formation, namely the syncytial blastoderm stage, several types of monster are produced from most of the centrifuged eggs by strong centrifugation above 3,500 g.; these are again correlated with the direction of centrifugation.

After posterior centrifugation, larvae are obtained in which several abdominal segments from the caudal end are missing; after anterior centrifugation, larvae lack a head or a head plus some thoracic segments; and, finally, after oblique centrifugation, various transitional types occur (Plate 2, figs. J, K).

It is clear, therefore, that centrifugation during the syncytial blastoderm stage gives rise to defects along the body-axis and no longer produces duplication of parts.

If the same centrifugal force (over 3,500 g.) is applied a little later, at the stage in which the blastoderm cells have formed cell partitions at the sides only (but are still continuous with the yolk underneath), almost all of the treated eggs develop into another type of abnormality. In an extreme degree, although cell differentiation seems to have reached the final state, the cells are not organized into an embryo and the resulting condition suggests a random mixing of fragments of the normal embryo (Plate 2, figs. L, M, N). This probably corresponds to what Pauli described as 'unharmonisches Gewebe' (1927).

Though it is difficult to explain on a common basis the malformations produced by early and by late centrifugation, the changes of malformation type with developmental stage from early nuclear division to blastoderm formation make us visualize a course along which the egg proceeds from an indeterminate state to a 'mosaic' state through successive stages.

DISCUSSION

Two points will be taken up in this discussion. The first concerns specific features of the development of *C. dorsalis*, particularly the development of the thorax, and the second is the more general problem of the relation of the mode of development of *Chironomus* to that of other Dipterans.

In *Chironomus* the development of the thorax seems to be a highly perplexing point. The basic propositions involved in the phenomena will, therefore, be analysed and enumerated without any further intention of finding an explanation.

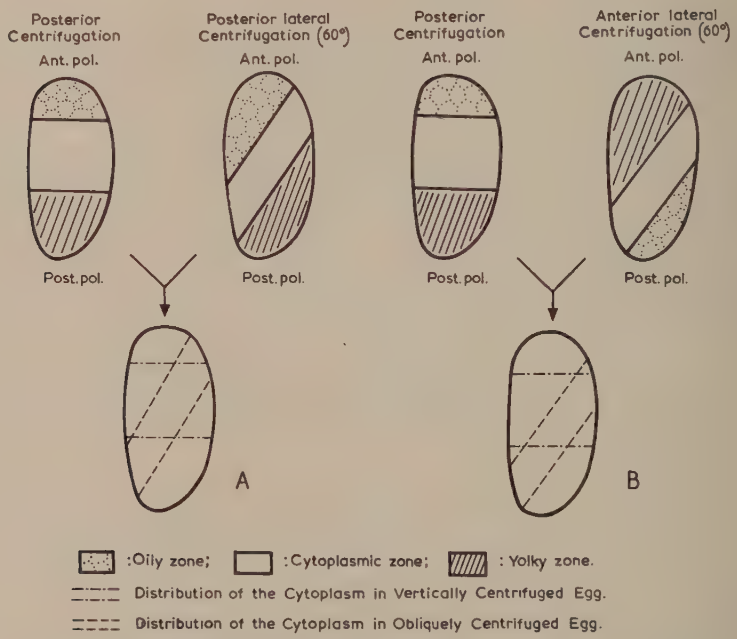
Discussing first the vertical centrifugation experiments, double cephalon results from posterior centrifugation and double abdomen follows anterior centrifugation. Three propositions may be derived from these results. Proposition (I): a locality having a gross tendency for head development must lie somewhere in the anterior part of the egg; proposition (II): a locality for abdominal development must lie somewhere in the posterior region; proposition (III): such localities must be carried by a substratum not movable by the centrifugal forces used in the present experiments. The fact that we can make these localities bring forth their final products selectively one at a time by supplying cytoplasm to the anterior or posterior side of the egg shows that they have definite and mutually independent existences. On the centripetal side, the border of the cytoplasmic zone is distant from the pole by one-fifth of the long axis, and on the centrifugal side by two-fifths the long axis of the egg (Plate 1, fig. A). The fact that the character of one pole determines that of the product while the other pole has no influence whatsoever, might indicate that the formative centres lie within one-fifth of the axial length from the poles of the egg and do not spread beyond.

Two more questions then follow. (*a*) What causes the doubleness of the structure? (*b*) Is it possible to find a similarly independent locality for the thorax in an intermediate region of the egg?

(*a*) Concerning the doubleness of the structure, the author thinks that this must be closely dependent on the mode of redistribution after centrifugation of the cytoplasm which proceeds symmetrically towards both poles. As the result of the head-determining and abdomen-determining localities noted above under (I) and (II), when the cytoplasm is shifted to one end of the egg its fate will be set towards head or towards abdomen. If such cytoplasm, the fate of which has been determined, spreads symmetrically towards the two poles covering the spacious surface area, it is not impossible to imagine the formation of a duplicate set of structures which are mirror images of each other (Waddington, 1956).

(*b*) Evidence for a thorax-locality is entirely lacking. As was described in section (3), in the posterior centrifuged egg, although the transparent cytoplasm comes mostly to the lighter (anterior) hemisphere, its centrifugal border goes beyond the equator, invading the heavy half. To be more explicit, the cytoplasm extends between the levels at one-fifth and three-fifths along the long axis of the egg as measured from the micropyle end. In anterior centrifugation the cytoplasm occupies the levels between one-fifth and three-fifths along the long axis from the opposite end of the egg; the middle zone (two-fifths to three-fifths) is, therefore, cytoplasm in both cases. Yet in neither case is the thorax formed, the former producing purely cephalic, and the latter purely abdominal structures. In other words, there is no particular intermediate zone to be allocated to the thorax.

The thorax is met with for the first time in oblique centrifugation. Although the monsters resulting from oblique centrifugation still carry a reminiscence of vertical centrifugation by having caps consisting of a pair of heads or a pair of caudal segments at the two ends, a thorax has been interposed between them. In lateral centrifugation, as was stated before, all the treated embryos are normal, having thoraces.


Posterior Centrifugation
Ant. pol.
Post. pol.
Posterior lateral Centrifugation (60°)
Ant. pol.
Post. pol.
Posterior Centrifugation
Ant. pol.
Post. pol.
Anterior lateral Centrifugation (60°)
Ant. pol.
Post. pol.
A
B
:Oily zone; :Cytoplasmic zone; : Yolky zone.
Distribution of the Cytoplasm in Vertically Centrifuged Egg.
Distribution of the Cytoplasm in Obliquely Centrifuged Egg.

TEXT-FIG. 4. Superimposed sketches of two kinds of stratification (see text). *Ant. pol.*, *Post. pol.*, anterior and posterior poles of the egg respectively. A, posterior centrifugation and posterior-lateral centrifugation. B, anterior centrifugation and posterior-lateral centrifugation.

Unfortunately, the explanation of oblique centrifugation seems to be very complicated, and even enumeration of the basic conditions for the realization of such a result is impossible.

The only obvious difference between vertical and oblique centrifugations is that in the latter case the transparent zone extends farther towards the two poles of the egg. But such a quantitative difference alone can hardly be an adequate explanation for such a fundamental qualitative change as the appearance of the thorax.

Compare, for instance, posterior centrifugation and posterior-lateral centrifugation. The former gives rise to a double cephalon and the latter gives rise to a partial embryo with a perfect head, a perfect thorax, a portion of the abdomen, and a part of a reversed head. In the latter experiment, the hyaline zone covers the levels occupied by the hyaline protoplasm in the vertical centrifugation and extends beyond it at the two ends (Text-fig. 4A). Considered naïvely, this situation might even suggest a strengthening of head and caudal development, but there is no positive factor to foreshadow the formation of the thorax.

As another example, posterior centrifugation may be compared with anterior-lateral centrifugation. In contrast to the double cephalon which the former gives rise to, the latter causes the production of a partial embryo with a perfect abdomen, a complete thorax, and a partial head, and reversely directed caudal segments. In other words, in anterior-lateral centrifugation, since the hyaline zone is far removed from the anterior end of the egg, very little of the head is formed in the resulting embryo. Yet the hyaline zone in the latter case extends still farther towards the anterior end of the egg than in the posterior (vertical) centrifugation (Text-fig. 4B). All these facts indicate that a simple topographical scheme postulating head or tail organizing areas at the two ends of the egg is not adequate to explain the result of oblique centrifugation. Probably a new hypothesis, such as a neutralization or interference between the effects of two centres, may be required to explain the formation of the thorax. In this connexion we think of the double gradients in sea-urchin development. However, the present state of our knowledge of harlequin-fly development is inadequate to make any judgement as to whether the idea is applicable or not. Consequently, proposition (IV) is tentatively suggested: when the activities of the two centres meet, some kind of interference takes place which leads to the formation of the thorax.

The effects of reducing the blastoderm size, described in section (5), can be covered without introducing any more factors, since these results simply serve as another example of the priority of the terminal characters over the intermediate zones.

An obvious feature of the results of two successive centrifugations, one given at the nuclear division stage and the other after the syncytial blastoderm stage, is the change of doubleness of the resulting structure into singleness. This change must certainly be caused by the delay of the second centrifugation. If the formative influence from the two centres retains its activity until the second centrifugation is given, the result of such a treatment would not differ from that of two centrifugations given close together during the nuclear division stage—i.e. a double structure. The loss of doubleness must be partly due to the fact that these centres cease to be active sometime between the nuclear division stage and the syncytial blastoderm stage, before the second centrifugation is given (proposition V). In other words, by the first centrifugation, given during the nuclear division stage, the cytoplasm is charged with a tendency towards head or abdominal development. Then it redistributes itself in symmetrical fashion. By the time the syncytial blastoderm stage is reached and the second centrifugation is given, the centres have lost their activity. As the result, the sole meaning of the second centrifuging is a re-establishment of one-sided accumulation of the cytoplasm. Such a late amassing of the cytoplasm will shorten the time available for its redistribution before definitive structures are formed, which results in the singleness of structure.

Centrifugation at the syncytial blastoderm stage for the first time gives rise to embryos which lack a few segments at one end of the body. The missing end always corresponds to the centrifugal end. Within the range of proposition (V) the following tentative interpretation is possible. By the syncytial blastoderm stage, the determination of the head, thorax, and abdomen must be advancing before the cytoplasm at the centripetal side is shifted down by one-fifth the axial length while the cytoplasm at the centrifugal side is pushed up by two-fifths of this distance. It can be suggested that the omission of the centrifugal segments may be due to the fact that the final structures are determined before the return of the cytoplasm to the farthest centrifugal end or to some kind of interaction with the influence of the opposite pole.

In this series of experiments the persistence of the supposedly labile thorax, even after the elimination of a supposedly more firmly established centrifugal terminal character, might seem rather odd. But this is not necessarily a contradiction because, however labile the thorax determination is, once it is established, as an intermediately situated organ, the thorax will be more favoured than end structures, since centrifugation results in accumulation of cytoplasm in the middle part of the egg, causing least disturbance in the thorax region.

Recapitulating the propositions:

(I) A locality having a gross tendency towards head development is located in the anterior end of the egg.
(II) A locality having a gross tendency towards abdominal development lies in the posterior end of the egg.
(III) Such localities must be carried by a substratum not movable by centrifugal forces of the magnitudes used in this experiment.
(IV) Some kind of interaction must take place between the influences of the two poles, and when this happens, a thorax results.
(V) The formative activities of the centres are lost by the syncytial blastoderm stage.

Concerning (III), in flies and beetles a superficial cytoplasm or *Keimhautblastem* has been observed, which is supposed to be the locus of determination. Recently Kaudewitz (1950) obtained, in the egg of *Daphnia*, malformations related to the direction of centrifuging and suggested the presence of an immovable cortex in which determination is carried. In the *Chironomus* egg, however, microscopical observations of both living and sectioned eggs shows that a cytoplasmic layer corresponding to the so-called *Keimhautblastem* had been shifted by centrifugation, and the author believes there is another really immovable layer outside the *Keimhautblastem*.

Child (1943) reported a reduction-oxidation gradient in the ovarian egg of *Drosophila*. Apart from such an internal gradient, a diffusion gradient of substances such as oxygen or water or excretion substances can be imagined for some insect eggs which are provided with a hydropyle, as Costello (1948) once pointed out for marine eggs. But the best these facts can account for is a single gradient, which is not adequate to explain the development of *Chironomus*.

We have now reached a point where it is necessary to consider a general aspect of the development of *Chironomus*. From what has been said it may be clear that the cytoplasm and nuclei as a whole are labelled with gross characters such as head or abdomen at about the nuclear migration stage. During the syncytial blastoderm stage embryonic determination is pushed a step towards the mosaic side and finally, by the blastoderm stage, the embryo is almost completely mosaic. Customarily, the term 'determination time' in insect eggs is used to denote the time at which the egg reaches a mosaic condition. If so, it can be said that the determination time of *Chironomus* occurs much later than that of the house-fly and the fruit-fly. It has been generally believed that the determination time of Diptera is very precocious on the basis of the development of the house-fly and the fruit-fly. If the *Chironomus* egg is placed on the indeterminate–determinate series of insect eggs as suggested by Seidel (1936), it may come close to the Odonata which have been thought to be one of the most indeterminate groups. In this sense, it may become necessary in future to make a clear distinction between the lower and higher members of the Diptera.

A large difference in the degree of determination during the early period between the house-fly and *Chironomus* is further attested by comparing Pauli's results with those of the author. Among the abnormalities which Pauli obtained by treating *Musca* and *Calliphora* eggs shortly after oviposition, some defects along the body axis were found which bear a resemblance to the abnormalities of *Chironomus* centrifuged at the syncytial blastoderm stage. Of particular interest is Pauli's so-called 'unharmonisches Gewebe', which looks like the disorganized tissue masses of *Chironomus*. In spite of a close similarity between the two malformations, the former is obtained by the treatment of newly deposited eggs of *Musca* and of *Calliphora*, while the latter is produced by centrifugation of *Chironomus* at the late blastoderm stage.

Seidel, Bock, & Krause (1940) contend that the mode of embryonic determination can be closely correlated with the type of egg as classified by the localization of the germ band, the volume of the egg constituents, &c. However, as far as egg type is concerned, the *Chironomus* egg does not differ much from that of the house-fly and the fruit-fly except in quantity of cytoplasm. It may be very difficult to attribute the difference in the determination time to a simple difference in the quantity of the cytoplasm.

SUMMARY

1. By centrifuging, alone or in combination with constricting and puncturing of the egg, the embryonic determination in the harlequin-fly *C. dorsalis* has been studied.

2. In eggs centrifuged moderately, three zones were distinguished: a centrifugal yolk zone, a centripetal oil zone, and an intermediate zone consisting of cytoplasm with nuclei.

3. The mode of redistribution of the constituents of the centrifuged eggs differs according to the developmental stage at the time of centrifugation and to the centrifugal force. The strata of weakly centrifuged eggs begin to mix with each other immediately after centrifugation. Redistribution of the constituents of strongly centrifuged eggs always occurs in a definite way, even though the redistribution after centrifuging at the nuclear division stage does not take place until later, in the nuclear migration stage, while the cytoplasm of eggs centrifuged during and after nuclear migration begins to migrate as soon as the centrifuge is stopped.

4. By centrifuging the eggs at the nuclear division stage, various types of malformation related to the direction of centrifugation are caused. These malformations are characterized by having duplicated parts.

5. The frequency of occurrence of the malformations increases with increase in the centrifugal force.

6. The structure of the double monsters is further modified by separating a part of the yolk or oil in the centrifuged eggs by strong centrifuging or by constricting, and by removing a part of the cytoplasm from the centrifuged eggs by puncturing. Modification always occurs in the mid-part of the double monster in the form of loss of segments. The number of omitted segments depends on the volume of the separated or the removed part.

7. By applying two successive centrifugations in reverse directions it was found that a causal relationship between the direction of centrifugation before nuclear migration and the resulting malformation type is established at about the nuclear migration stage.

8. From late centrifugation, two other groups of malformations resulted which are different from those obtained by early treatment. Those of the first group, which were caused by centrifuging at the syncytial blastoderm stage, are characterized by having a defect on the centrifugal side and no duplicated parts. Those of the second group were obtained from eggs centrifuged at the cellular blastoderm stage. In the extreme degree of this malformation the cells which reach the final level of differentiation are not organized into an embryo, forming instead small tissue masses.

9. The specific features of the embryonic development of *C. dorsalis*, particularly thorax development, and the more general problem of the relation of the mode of development of *Chironomus* to that of other Dipterans, are discussed.

RÉSUMÉ

Recherches sur la détermination embryonnaire de la mouche-arlequin, Chironomus dorsalis. I. *Effets de la centrifugation et de sa combinaison avec les expériences de ligature et de ponction*

1. La détermination embryonnaire de la mouche-arlequin *Chironomus dorsalis* a été étudiée par la méthode de la centrifugation, seule ou combinée, à des ligatures et à des ponctions de l'œuf.

2. Dans des œufs centrifugés modérément, 3 zones peuvent être distinguées: une zone vitelline centrifuge, une zone huileuse centripète, et une zone intermédiaire comprenant le cytoplasme avec les noyaux.

3. Le mode de redistribution des constituants des œufs centrifugés diffère suivant le stade du développement au moment de la centrifugation et la force centrifuge. Les sédiments des œufs faiblement centrifugés commencent à se mélanger immédiatement après la centrifugation. La redistribution des constituants des œufs centrifugés fortement se fait toujours d'une manière bien définie, bien que la redistribution dans les œufs centrifugés au stade de la division cellulaire n'ait lieu que plus tard, tandis que le cytoplasme d'œufs centrifugés pendant et après la migration cellulaire commence à se déplacer sitôt que la centrifugation a pris fin.

4. Si l'on centrifuge les œufs au stade de la division nucléaire, on obtient différents types de malformations en rapport avec la direction de centrifugation. Ces malformations sont caractérisées par des dédoublements.

5. La fréquence des malformations augmente avec l'intensité de la force centrifuge.

6. La structure des monstres doubles est modifiée si l'on sépare de l'œuf une partie du vitellus ou de l'huile par une forte centrifugation ou par une ligature. Il en est de même si l'on enlève par ponction une partie du cytoplasme des œufs centrifugés. La modification se produit toujours dans la partie moyenne du monstre double, sous la forme d'une perte de segments. Le nombre de segments perdus dépend du volume de la partie isolée ou enlevée.

7. Quand on procède à deux centrifugations successives dans des directions opposées, on constate qu'une relation causale entre la direction de centrifugation avant la migration nucléaire et le type de malformation résultante s'établit environ au stade de la migration des noyaux.

8. Les centrifugations tardives donnent lieu à deux autres groupes de malformations différentes de celles qu'on obtient par un traitement précoce. Celles du premier groupe, qui sont provoquées par la centrifugation au stade du blastoderme syncytial, sont caractérisées par une déficience du côté centrifuge et par l'absence de dédoublements. Celles du deuxième groupe ont été obtenues à partir d'œufs centrifugés au stade du blastoderme cellularisé. Dans le cas extrême de ce type de malformations, les cellules qui atteignent le niveau final de différenciation

ne s'organisent pas en un embryon, elles forment à sa place de petites masses de tissus.

9. La discussion concerne: (*a*) Les traits caractéristiques du développement embryonnaire de *Chironomus dorsalis*, en particulier le développement du thorax; (*b*) le problème plus général du mode de développement du Chironome par rapport au développement des autres Diptères.

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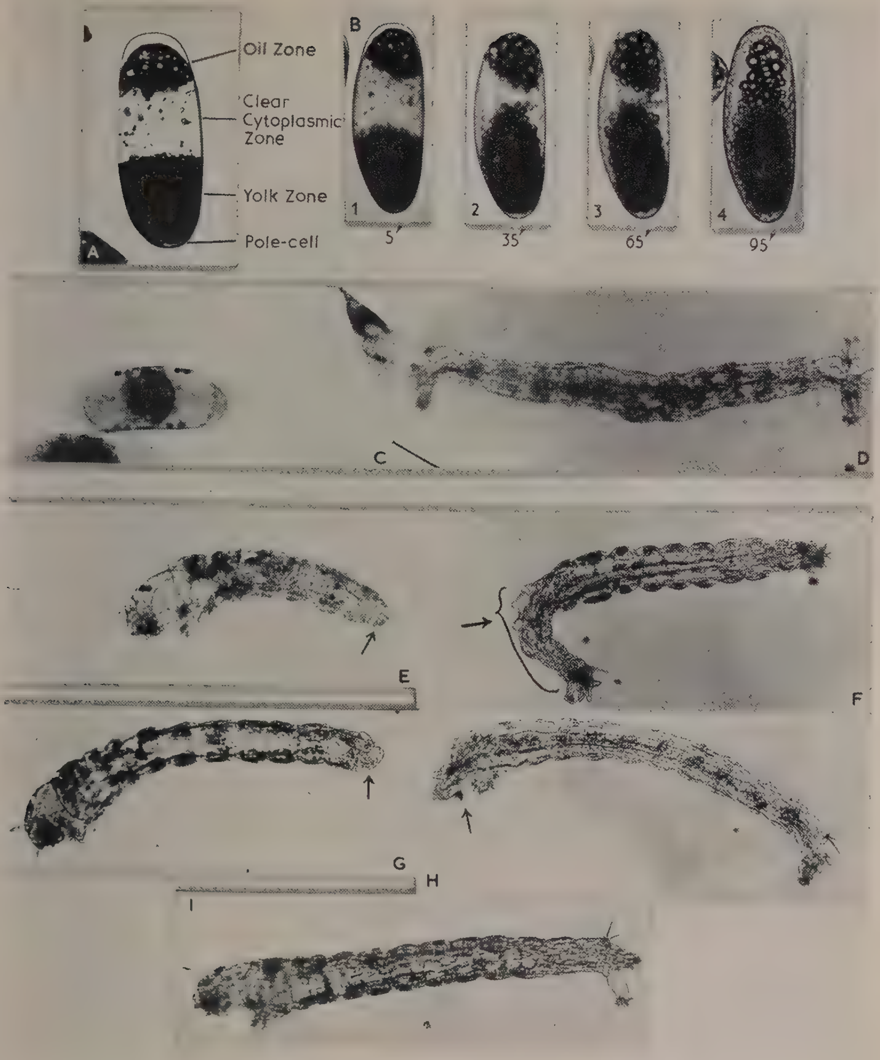
EXPLANATION OF PLATES

PLATE 1

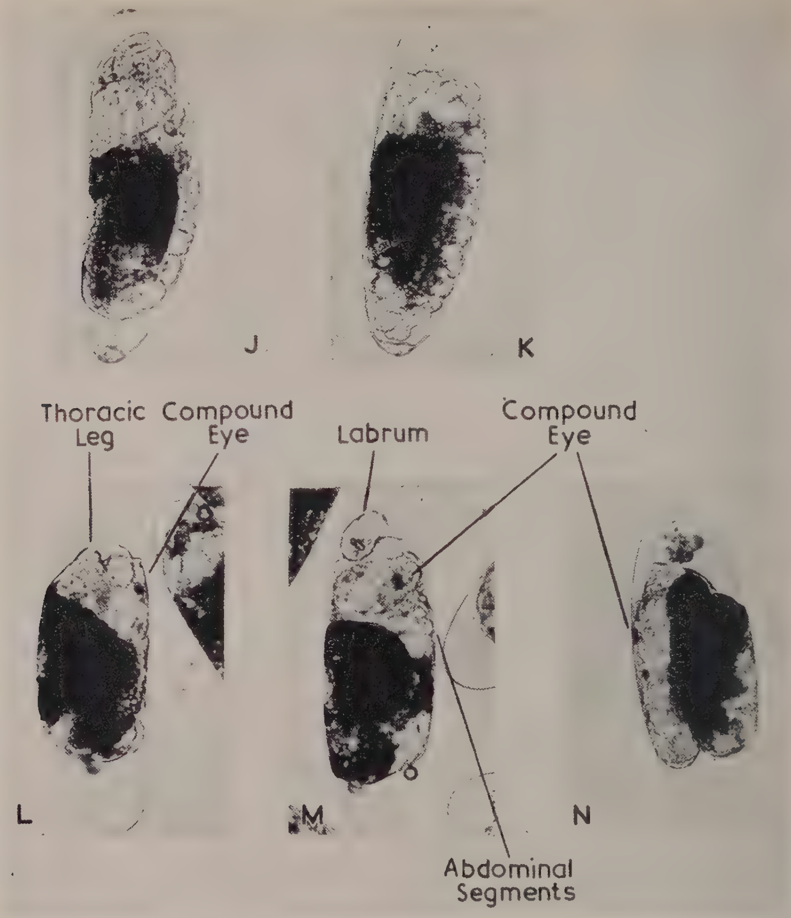
FIG. A. Stratification of the centrifuged eggs of *C. dorsalis* (3,000 g.) at nuclear division stage.

FIG. B. Redistribution of the constituents of the centrifuged egg at early stage. Time in minutes after nuclear migration stage at 25° C.



H. YAJIMA

Plate 1



H. YAJIMA

Plate 2

FIGS. C, D. Malformations obtained by vertical centrifugations.

FIG. C. Double cephalon obtained by posterior centrifugation (see text).

FIG. D. Double abdomen resulting from anterior centrifugation (see text).

FIGS. E–H. Monsters obtained by oblique centrifugations. Arrows indicate minor parts produced in an abnormal direction.

FIG. E (60°) and FIG. G (30°) show malformations following posterior-lateral centrifugation. (Cf. Table 1.)

FIG. F (60°) and FIG. H (45°) show malformations obtained by anterior-lateral centrifugation. (Cf. Table 1.)

FIG. I. Normal larva resulting from lateral centrifugation.

PLATE 2

FIGS. J and K show monsters produced by strong centrifugation at the syncytial blastoderm stage.

FIGS. L–N. Malformations (disorganized tissue masses) obtained by strong centrifugation at early cellular blastoderm stage.

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Étude immuno-électrophorétique de l'apparition progressive de quelques constituants caractéristiques de l'adulte dans le foie embryonnaire du Poulet

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DANS le cadre des recherches consacrées à l'étude de l'apparition des constituants spécifiques de l'adulte au cours du développement embryonnaire, les méthodes immunochimiques ont trouvé un emploi toujours grandissant pendant les 15 dernières années. Le nombre de travaux, dont les principaux ont été récemment exposés (Croisille, 1958), permet de juger combien l'emploi de ces méthodes s'est révélé fructueux dans ce domaine. La plupart des auteurs ont utilisé les méthodes immunochimiques classiques: réaction à l'interface, épuisement des anti-sérums, techniques de précipitation en milieu gélifié et réaction de déviation du complément. Mais ces méthodes ne permettent souvent pas, malgré leur spécificité et leur sensibilité, de dénombrer et d'identifier correctement tous les constituants, surtout s'il s'agit de l'analyse d'un mélange complexe. Aussi y a-t-il des résultats contradictoires dus pour la plupart à des difficultés techniques ou d'interprétation, notamment en ce qui concerne l'identification certaine des constituants embryonnaires et adultes.

Dans l'étude présente, nous nous sommes adressé à une méthode nouvelle, la technique d'immuno-électrophorèse de Grabar & Williams (1953, 1955) qui utilise à la fois la migration électrophorétique et la précipitation spécifique en milieu gélifié. Cette méthode permet donc de caractériser chaque constituant d'un mélange par une double définition, sa mobilité électrophorétique et ses propriétés immunochimiques.

Grâce à l'emploi de cette technique, nous avons pu suivre l'apparition progressive de quelques constituants caractéristiques de l'adulte dans le foie de l'embryon de Poulet.

MATÉRIEL ET TECHNIQUE

Préparation des extraits d'organes

Les extraits utilisés dans cette étude proviennent d'organes prélevés sur des Poulets (adultes ou embryonnaires) de la race Leghorn blanc. Les foies prélevés

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ont été coupés en petits morceaux et lavés dans du liquide de Tyrode. Ensuite, ils ont été broyés dans un mortier avec du sable jusqu'à obtention d'une masse homogène. Le broyat a été repris dans du liquide de Tyrode à la température de 5° C. dans les proportions suivantes: 1 $cm.^3$ de Tyrode pour 1 g. de tissu, pour le foie adulte; et 1 $cm.^3$ de Tyrode pour 5 g. de tissu pour les extraits de foie embryonnaire.

Après centrifugation à 10.000 t.p.m. (environ 10.000 g.) pendant 15 à 20 minutes, l'extrait obtenu a été maintenu entre — 10 et — 15° C. jusqu'à utilisation.

Préparation de l'immunsérum

Pour préparer le sérum anti-foie adulte (*SAF*), nous avons injecté de l'extrait de foie de Poulet adulte à 6 lapins mâles âgés de 6 à 8 mois, au rythme de 2 à 3 injections par semaine. Chaque injection a été partagée en 2 $cm.^3$ par voie intrapéritonéale et 1 ou 2 $cm.^3$ par voie intraveineuse. Après 3 mois, nous avons prélevé 15 $cm.^3$ de sang par ponction cardiaque. Les sérums des 6 lapins ont donné un ring-test positif avec l'extrait de foie adulte. Cependant 3 lapins seulement avaient produit un nombre satisfaisant d'anticorps. Le mélange de ces 3 sérums a été retenu pour faire quelques études préliminaires. Après un mois de repos, les 3 lapins sélectionnés ont reçu 7 nouvelles injections en 15 jours. Le sérum recueilli 10 jours après la dernière injection avait un pouvoir précipitant et un nombre d'anticorps nettement plus élevé. Les résultats exposés dans cette étude ont été obtenus avec le mélange de ces derniers sérums. Le détail de la progression de l'immunisation sera exposé dans un prochain travail.

Techniques utilisées

Double diffusion en plaques de gélose

Nous avons effectué quelques essais en utilisant la technique de double diffusion d'Ouchterlony (1948). L'antigène et l'immunsérum liquides sont versés dans des trous préparés dans une plaque de gélose; ainsi les deux réactifs diffusent librement dans le gel et, lorsqu'ils se rencontrent en proportions convenables, forment une ligne ou bande de précipité. Mais il arrive que les lignes de précipitation se superposent ou se dissocient, ce qui rend leur dénombrement et leur identification difficiles et souvent impossibles.

Immuno-électrophorèse

La méthode de Grabar & Williams (1953) consiste à effectuer une électrophorèse du mélange à étudier dans un gel de gélose. Lorsque la dispersion des constituants est jugée suffisante, on fait diffuser l'immunsérum perpendiculairement à l'axe de migration. Chaque constituant donnant une fine bande de précipité avec l'anticorps correspondant, on peut le définir par son emplacement sur l'axe de migration. Le nombre de lignes ainsi obtenues correspond au nombre minimum de constituants présents dans le mélange. Nous avons utilisé cette méthode dans les conditions expérimentales suivantes:

— gélose à la concentration de 1 pour cent et sous une épaisseur de 3 à 4 mm.,
— tampon véronal sodique de pH 8,2 et $\mu = 0{,}05$,
— potentiel de 4 à 5 volts/cm.,
— durée de l'électrophorèse: $3\frac{1}{2}$ heures,
— distance entre l'antigène et l'immunsérum liquide: 5 à 6 mm.

Les plaques sont maintenues en chambre humide à la température du laboratoire et le développement des lignes de précipitation suivi et surveillé pendant 5 jours.

RÉSULTATS

Diffusion en milieu gélifié

En utilisant la méthode de double diffusion d'Ouchterlony, nous avons obtenu jusqu'à 5 zones de précipitation avec l'extrait de foie adulte. Leur dénombrement exact est cependant assez difficile; trois d'entre elles sont réellement nettes, les deux autres sont plutôt floues et semblent correspondre à la superposition de plusieurs lignes. Les trois systèmes principaux correspondent à un constituant à diffusion rapide (*X*), un constituant à diffusion lente (*Y*) et un constituant à diffusion moyenne (*Z*). Les lignes *X* et *Y* se forment avec l'extrait de foie adulte et les extraits de foie de poussin de 5 jours, d'embryons de 17, 14 et 10 jours. La ligne *Z* correspond à un constituant qui apparaît vers le 16$^{\text{ème}}$ jour de la vie embryonnaire.

Immuno-électrophorèse

Avec le mélange de sérum anti-foie adulte choisi, nous avons obtenu jusqu'à 16 lignes de précipitation distinctes pour l'extrait de foie de Poulet adulte. La nomenclature que nous avons adoptée (Fig. 1) est basée sur l'emplacement des lignes de précipitation en partant du pôle positif (+) vers le pôle négatif (—) de l'axe de migration. Nous avons en outre tenu compte des positions relatives des lignes les unes par rapport aux autres.

L'ordre d'apparition des différentes lignes est le suivant: tout d'abord se forment les lignes *A*, *C*, *D*, *E*, *E′*, *G* (pendant les 20 premières heures), puis apparaissent les lignes *B*, *E″*, *F*, *H*, *I* (pendant les 20 heures suivantes). Les autres apparaissent plus tardivement et assez irrégulièrement. Par leur position les 16 lignes de précipitation observées semblent toutes correspondre à des systèmes précipitants différents. En outre, elles finissent presque toutes par se croiser. La ligne *F* peut dans certains cas sembler correspondre à un dédoublement de la ligne *G*, mais, si on prolonge la durée de l'électrophorèse, elle peut être située au niveau de l'intersection des arcs *E* et *G*, ce qui montre bien qu'elle correspond elle aussi à un constituant distinct.

La distribution et le moment d'apparition des différents constituants pendant le développement sont résumés dans la Fig. 2 A, B. On voit que les constituants *A*, *B*, *C*, *D*, *E* et *G* sont déjà présents dans le foie de l'embryon de 6½ jours. Nous avons effectué deux expériences avec de l'extrait de foie d'embryons de 5 jours, et nous n'avons pu voir l'apparition que d'une seule ligne qui, par sa position, pourrait correspondre au constituant *E*. L'absence de réaction ne démontre pas l'absence des autres constituants. On peut penser plutôt qu'à ce stade leur concentration n'est pas suffisante pour former un précipité. Ne disposant que d'une quantité très minime de matériel à ce stade, nous n'avons pas pu concentrer l'extrait.

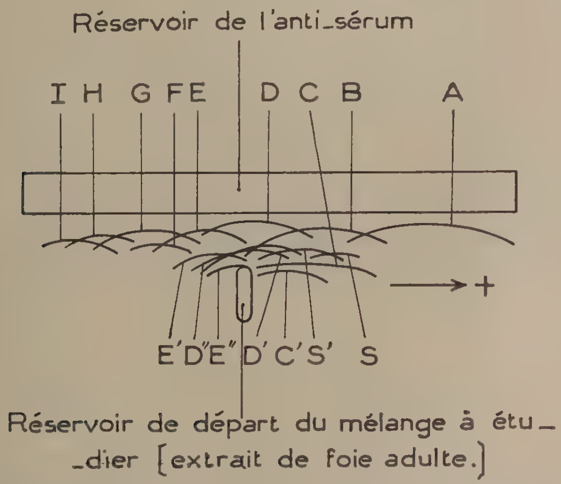
Réservoir de l'anti_sérum
I H G F E D C B A
→ +
E' D'' E'' D' C' S' S
Réservoir de départ du mélange à étu_dier [extrait de foie adulte.]

FIG. 1. Schéma et nomenclature des lignes de précipitation obtenues avec de l'extrait de foie adulte en présence de sérum anti-foie de Poulet adulte.

TABLEAU 1

Apparition progressive des constituants adultes pendant le développement embryonnaire

Constituants: *Stades*	*A*	*B*	*C*	*C'*	*D*	*D'*	*D''*	*E*	*E'*	*E''*	*F*	*G*	*H*	*I*
Foie adulte	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Foie de poussin 5 jours . .	+	+	+	+	+	—	—	+	+	—	+	+	+	+
Foie embryonnaire 17–18 jours .	+	+	+	—	+	—	—	+	—	—	+	+	+	+
,, 13–14 jours .	+	+	+	—	+	—	—	+	—	—	+	+	—	+
,, 8–9 jours .	+	+	+	—	+	—	—	+	—	—	—	+	—	+
,, 6½ jours .	+	+	+	—	+	—	—	+	—	—	—	+	—	—
,, 5 jours . .	—	—	—	—	—	—	—	+ ?	—	—	—	—	—	—

Notons qu'aucune réaction n'a pu être observée avec l'extrait de foie de Souris adulte ou embryonnaire. Les constituants correspondants aux lignes de précipitation observées, semblent donc être liés à l'espèce étudiée ou au moins à la classe des Oiseaux. De nouvelles expériences devront être faites avec des extraits d'organes de Canard, par exemple, pour préciser cette question.

A l'intérieur de l'espèce, nous avons observé de nombreuses réactions avec le sérum et d'autres extraits tels que l'extrait de cerveau, d'intestin, de peau, de mésonéphros, de métanéphros, de poumon et de cœur. Nous avons aussi noté des réactions avec les jus d'embryons de 5 jours et de 3 jours et avec le jaune d'œuf et le blanc d'œuf.

Dans ce travail, nous n'étudierons que les réactions obtenues avec le sérum de Poulet adulte (*SP*). Comme le montre la figure 3, le sérum de Poulet donne un certain nombre de lignes au contact du *SAF*. En absorbant le sérum anti-foie adulte par du sérum de Poulet adulte (*SAF/SP*), il ne réagit plus avec aucun

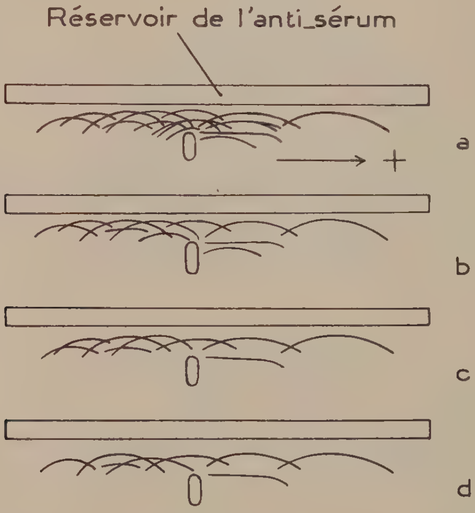

FIG. 2A

FIG. 2 A, B. Étude comparative des extraits de foie de Poulet adulte et embryonnaire à différents stades du développement. Les rectangles parallèles aux axes de migration électrophorétiques représentent les réservoirs dans lesquels a été versé le sérum anti-foie adulte. Les lignes de précipitation n'ont été figurées que d'un seul côté pour éviter de surcharger les schémas qui sont donnés en grandeur réelle.

(*a*) *Foie de Poulet adulte*: en présence de sérum anti-foie on obtient 16 lignes de précipitation distinctes. (Pour la nomenclature, voir Fig. 1.) La plupart de ces lignes se croisent, ce qui montre que les constituants correspondants n'ont aucune parenté immunologique.

(*b*) *Foie de poussin de 5 jours*: on observe 11 lignes. En dehors des arcs *S* et *S'*, il manque les arcs *D'*, *D''* et *E''*.

(*c*) *Foie d'embryons de 17–18 jours*: on note la présence de 9 lignes. Il manque les arcs *S*, *S'*, *C'*, *D'*, *D''*, *E'* et *E''*.

(*d*) *Foie d'embryons de 13–14 jours*: il manque les arcs *S*, *S'*, *C'*, *D'*, *D''*, *E'*, *E''* et *H*.

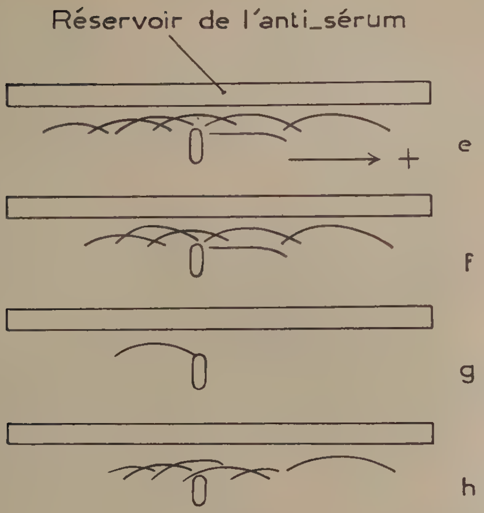

FIG. 2B

(*e*) *Foie d'embryons de 8–9 jours*: on peut observer 7 lignes de précipitation qui correspondent aux constituants *A*, *B*, *C*, *D*, *E*, *G* et *I*.

(*f*) *Foie d'embryons de 6½ jours*: sont présents les arcs *A*, *B*, *C*, *D*, *E* et *G*.

(*g*) *Foie d'embryons de 5 jours*: seul l'arc *E* est présent. L'absence de réaction des autres constituants n'indique pas forcément leur absence totale; ils peuvent être présents à des concentrations trop faibles pour réagir.

(*h*) *Extrait de tronc d'embryons de 5 jours* (noter la présence de l'arc *A*).

constituant du *SP*, ni avec les constituants *S–S'* et *A* du foie adulte. Tous les autres constituants du foie réagissent avec *SAF/SP*, ce qui montre qu'ils sont différents des constituants sériques. Dans toutes les expériences d'absorption, nous avons employé, comme témoin, du *SAF* auquel nous avions ajouté la même quantité de liquide physiologique qu'il était nécessaire d'ajouter de sérum de Poulet pour obtenir une absorption complète (*SAF/T*).

En dehors des constituants *S* et *S'* qui paraissent être des constituants du sérum, l'absence de réaction du constituant *A* du foie avec le *SAF/SP* pose un problème particulier. En effet, le fait que la ligne *A* du foie se forme toujours à quelque distance du réservoir anticorps, alors que la ligne correspondante du sérum se forme toujours au contact direct de ce réservoir, suggère que les 2 molécules ne sont pas identiques, n'ayant pas le même coefficient de diffusion. Mais ce résultat pouvant être dû à une différence de concentration d'un même constituant dans les deux milieux, ne suffit pas pour établir une différence entre les deux molécules. Aussi de nouvelles recherches étaient-elles nécessaires pour préciser ce problème. Là encore, la méthode immuno-électrophorétique devait se révéler très fructueuse. Il est en effet possible, une fois l'électrophorèse terminée, de découper des cubes de gélose aux endroits où se trouvent les constituants que l'on se propose de comparer, et de les transporter au contact de l'immunsérum dans une autre plaque de gélose, en réalisant ainsi une expérience de double diffusion, suivant le schéma donné par Ouchterlony (1948). Ceci permet la comparaison directe de deux constituants séparés par l'électrophorèse. D'autre part, comme on se trouve en présence d'un seul ou d'un nombre très restreint de systèmes précipitants, le dénombrement des lignes est très aisé et l'étude de leur évolution largement facilitée. Ainsi, nous avons pu comparer les différents constituants du foie adulte et embryonnaire entre eux et avec ceux du sérum et d'autres extraits d'organes, pour lesquels nous avions observé des réactions croisées. Cette étude est en cours et fera l'objet d'une prochaine note. Disons simplement ici que les résultats préliminaires montrent une réaction d'identité complète entre le constituant *A* du foie et le constituant correspondant du sérum.

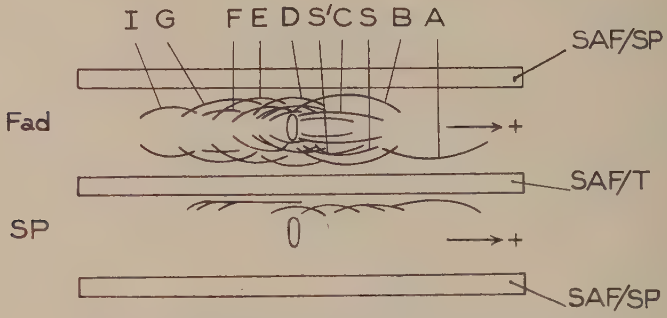

FIG. 3. Schéma des lignes obtenues avec l'extrait de foie adulte (*Fad*) et le sérum de Poulet adulte (*SP*), en présence de sérum anti-foie adulte (*SAF/T*) et de sérum anti-foie absorbé par le sérum de Poulet (*SAF/SP*). On voit que *SAF/SP* ne réagit plus avec *SP* ni avec les constituants *S–S'* et *A* du foie. L'absence de réaction du constituant *H* en présence de *SAF/SP* pose un problème particulier: soit que ce constituant est présent dans l'extrait de foie et dans le sérum, soit que dans l'antisérum absorbé les anti-corps anti-*H* sont en concentration trop faible pour réagir. En effet, avec un sérum anti-foie témoin auquel on a ajouté la même quantité de liquide de Tyrode qu'il était nécessaire d'ajouter de sérum de Poulet pour obtenir une absorption complète (*SAF/T*), le constituant *H* ne réagit pas.

Par la même technique, nous avons comparé séparément les différents constituants du foie adulte avec leurs correspondants embryonnaires, et nous avons observé qu'ils donnent tous lieu à des réactions d'identité complète.

Il convient d'ajouter ici que dans certaines expériences d'immuno-électrophorèse, nous avons employé du sérum anti-foie adulte absorbé par un extrait de foie d'embryons de 9 jours (*SAF/F*9). Nous avons observé que ce sérum ne réagit plus avec aucun constituant des extraits de foie embryonnaires de 9 jours et de 7 jours. En présence d'extrait de foie embryonnaire de 13 jours il donne une seule ligne de précipitation qui, par son emplacement sur l'axe de migration, correspond au constituant *F*. Or on sait que, dans nos expériences, le constituant *F* est le seul qui différencie le foie embryonnaire de 13 jours du foie embryonnaire de 9 jours. De même en présence d'extrait de foie adulte, *SAF*/*F*9 ne réagit qu'avec les constituants qui différencient le foie adulte du foie embryonnaire de 9 jours, c'est-à-dire *C'*, *D'*, *D''*, *E'*, *E''*, *F* et les deux constituants sériques *S* et *S'*. Il faut noter que le constituant *H* ne réagit pas. Mais il en est de même dans toutes les expériences d'absorption (Fig. 3). Nous pensons que dans les sérums épuisés (*SAF*/*F*9; *SAF*/*SP*; *SAF*/*T*) les anticorps anti-*H* sont en concentration trop faible pour réagir. Ces résultats vérifient pleinement l'identité des constituants embryonnaires et adultes et confirment leur ordre d'apparition pendant le développement.

Toutes les réactions croisées n'ayant pas encore été étudiées, il est difficile de déterminer dans quelle mesure les différents constituants sont porteurs de la spécificité d'organe. Néanmoins, il semble que les lignes *C'*, *D'*, *D''*, *E'*, *E''*, *F*, *H* et *I* correspondent à des constituants spécifiques du foie, car elles ne se rencontrent nulle part ailleurs, du moins si l'on en juge par leur position sur l'axe de migration électrophorétique.

DISCUSSION

L'inventaire des substances caractéristiques de l'adulte chez l'embryon a déjà souvent été fait à l'aide des méthodes immunochimiques classiques. Mais il subsiste des doutes quant à l'identification certaine des constituants embryonnaires et adultes. En effet, la réaction d'une protéine embryonnaire avec un anticorps produit en réponse à l'administration d'une protéine de l'adulte, n'établit pas forcément l'identité des deux protéines. On comprend ainsi l'avantage que présente, dans ces études, la méthode d'immuno-électrophorèse de Grabar & Williams (1953). En dehors de la réaction de précipitation spécifique, elle permet de définir les différents constituants d'un mélange par une propriété physique, leur mobilité électrophorétique. Elle facilite d'autre part le dénombrement des lignes de précipitation et, dans tous les cas, elle permet de révéler un nombre plus grand de constituants que toutes les autres méthodes immunochimiques.

Grâce à cette méthode, nous avons pu démontrer l'existence d'au moins 14 constituants différents dans le foie du Poulet adulte. Parmi ces constituants, 11 sont présents dans le foie du Poussin de 5 jours, 9 dans le foie de l'embryon de 17 à 18 jours, 8 dans le foie de l'embryon de 13 à 14 jours, 7 dans le foie de l'embryon de 8 à 9 jours, et 6 à $6\frac{1}{2}$ jours. Au $5^{\text{ème}}$ jour, nous n'avons pu voir qu'une seule ligne qui semble correspondre au constituant *E*. L'absence, à ce stade, du constituant *A* dans le foie est surprenante, car ce constituant est présent dans l'extrait de tronc d'embryons de 5 jours. On peut penser que dans l'extrait de foie de 5 jours, sa concentration n'est pas suffisante pour donner lieu à la réaction de précipitation. Ne disposant que d'une quantité minime de matériel, nous n'avons pas pu concentrer l'extrait.

Cette étude montre que les substances de l'adulte apparaissent progressivement au cours du développement embryonnaire et qu'au moment de l'éclosion la structure chimique, principalement la structure protéique, caractéristique de l'adulte, est loin d'être réalisée. Ces résultats rejoignent les travaux de Kaminski & Durieux (1956) et de Scheidegger *et al.* (1956), qui ont montré que, chez l'Homme et le Poulet, le sang est encore incomplet aux premiers stades de la vie post-embryonnaire.

RÉSUMÉ

La méthode immuno-électrophorétique permet de démontrer l'existence d'au moins 14 constituants différents dans l'extrait de foie de Poulet adulte. Parmi ces constituants, 6 sont déjà présents chez l'embryon de 6½ jours. Les autres apparaissent progressivement au cours du développement. Il faut noter qu'au moment de l'éclosion, la structure chimique caractéristique de l'adulte est loin d'être réalisée. L'identité des constituants embryonnaires et adultes est basée sur les propriétés suivantes: (1) ils ont même mobilité électrophorétique et donnent une ligne de précipitation au même endroit sur l'axe de migration; (2) ils ont même coefficient de diffusion dans la gélose; (3) en plaques de double diffusion, ils donnent lieu à des réactions d'identité complète.

SUMMARY

The immuno-electrophoretic method makes it possible to demonstrate at least 14 different constituents in extract of adult fowl-liver. Of these constituents, 6 are already present in the 6½-day embryo. The others appear progressively during development. It is notable that at hatching the chemical structure of the adult is far from being realized. The identity of the embryonic and adult constituents is based on the following similarity of properties: (1) they have the same electrophoretic mobility and give a precipitation line at the same place on the migration axis; (2) they have the same diffusion coefficient in agar; (3) in double diffusion plates they give completely identical reactions.

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Je remercie M. le Professeur P. Grabar de l'amabilité avec laquelle il m'a toujours accueilli dans son laboratoire et des nombreux conseils qu'il m'a donnés pendant la réalisation de ce travail.

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Stimulating Action of Tissue Extracts on Regenerative Processes

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WITH TWO PLATES

INTRODUCTION

THE problem of regeneration links together the interests of theory and practice very closely, and undoubtedly it is one of the cardinal problems of modern biology. The search for possible methods of controlling regenerative processes, of stimulating selectively the restoration of the main functional components of a tissue, must always lead deeply into the study of the mechanisms of growth and development. The specific influence of intracellular substances on tissue growth and differentiation has been discussed repeatedly. Such substances are sometimes termed 'tissue organizers' (Levander, 1945). Levander (1945, 1956) has shown the possibility of forming muscle and bone from poorly differentiated connective-tissue elements either by the transplantation of the corresponding tissues or by the local injection of alcohol extracts of these tissues. Ectopic ossification was obtained in the same way by Lacroix (1945). Distantly and specifically acting 'stimulators' have been found in the pulp of rat teeth (Griffié & Velley, 1954). A specific influence of skin extracts on the regeneration of skin has been observed by Marsillii & Ciuti (1953). The liberation from macerates of specifically acting substances that decrease mitotic activity in a homologous organ when it is regenerating has been demonstrated by Saetren (1956) and confirmed by Steuart (see Ebert, 1958*b*). Others, however, have obtained some increase of the mitotic activity of regenerating liver after injection of liver homogenate into the abdominal cavity (Teir & Ravanti, 1953; Blomqvist, 1957). A stimulating influence of tissue extracts on the regeneration of homologous tissues was also shown by Tumanishvili, Jandieri, & Svanidze (1956*b*). Saetren himself (1956) obtained a stimulating effect when he used small quantities of macerates.

Such substances also influence embryonic differentiation. The stimulation of growth of an organ homologous to a transplanted one has been observed in chick embryos after transplantation to the chorio-allantois (Murphy, 1916; Ebert, 1954,

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1958*a*) and after injection into the area vasculosa (Weiss, 1947). Increase of mitotic activity in embryonic organs after the injection of suspensions of the corresponding embryonic organs into the blood-vessels of an embryo has also been described (Andres, 1955). Tissue extracts (Tumanishvili, Jandieri, & Svanidze, 1956*a*) and tissue homogenates (Walter, Allman, & Mahler, 1956) have shown similar effects. A stimulating influence of liver suspensions on the growth of tadpole liver has been found (Romanova, 1957). Probably the mutual influence of paired organs shown by many authors (Weiss, 1952; Saetren, 1956; Liosner, 1958) is due to the same substances.

Existing data are evidently contradictory with respect to the direction of action of the intracellular substances on the homologous structures. Rose (1955, 1958), introducing his concept of 'specific inhibition', thought that specific stimulation does not exist, but the published data hardly allow one to agree with such a view.

The data mentioned above, which I have not space to discuss in full, suggest that substances liberated by cells are important for the regulation of processes which take place in the tissues. Specifically acting intracellular substances are, I suggest, the most direct and primitive means of interaction between homologous cells and of influence by differentiated cells on relatively poorly differentiated cells. These substances may stimulate or suppress homologous cell reproduction and direct the differentiation of less differentiated elements.

The liberation of intracellular substances must proceed especially actively in the initial stage of regeneration because of the intensive cytolysis. This mechanism apparently takes part in the formation of the regenerate, influencing the direction of differentiation of the dedifferentiated tissue elements.

An attempt to use specifically acting intracellular substances to regulate regenerative processes seemed worth making. Since it was assumed that these substances can be transferred through the intercellular medium and blood, they should be extractable with physiological salt solutions. This assumption was confirmed completely in experiments with chick embryos (Tumanishvili, Jandieri, & Svanidze, 1956*a*). Such extracts have been used in the present experiments with the aim of getting specific stimulation of regeneration. The results have in part been published in preliminary communications (Tumanishvili, Jandieri, & Svanidze, 1956*b*; Tumanishvili, 1958*a*).

EXPERIMENTAL METHODS

The effect of tissue extracts on liver and muscle regeneration was studied. The main experiments were made with frogs (*Rana ridibunda*), but one group of experiments on liver regeneration was made with rabbits and guinea-pigs.

Preparation of the extracts

The extracts in all experiments were prepared in the same way. The tissue was ground thoroughly with quartz sand. For the injections into frogs Ringer solution or 0·65 per cent. sodium chloride or water was added to the resulting brei. For the injections into mammals the extraction was made with 0·9 per cent. sodium chloride solution. The ratio of tissue-weight to volume of added fluid was 1:2 or 1:1. All these operations were carried out on ice. The mixture was kept for an hour at a temperature of 2–4° C., and then filtered through compressed filter-paper. Such a filter is equivalent to a microbiological filter and the extract so obtained is practically free of cell elements. Membrane filters have recently been used and have given the same results, as also did the removal of suspended particles by centrifugation. The extract may be kept for several days at a temperature not higher than 2° C. By 5–7 days, however, there is an appreciable loss of its stimulating activity.

Arrangement of injections

In the experiments with frogs the injections were made post-operatively and then twice a day, every 12 hours. The daily dose at the beginning of the experiment was 0·4 ml., and it was increased by steps till it reached 1 ml. per day. In the experiments on stimulation of liver regeneration the extract was injected for 5 or 10 days, and in the experiments on muscle regeneration for 10, 18, or 27 days. The extract was injected intraperitoneally. In the rabbits and guinea-pigs the injections were made subcutaneously for 10 days, three times a day, 1·5 ml. at each injection with no subsequent increase in dose.

STIMULATION OF MUSCLE REGENERATION BY SALINE EXTRACTS

The frog gastrocnemius muscle was used in these experiments. A transverse incision into the muscle was made with a scalpel. The length of the incision was about 4 to 5 mm. and the depth about 3 to 4 mm. Because of the contraction of cut fibres and the separation of the wound edges, the wound acquired a funnel-like form, somewhat flattened at the proximal and distal sides.

The frogs were killed after 12, 21, or 30 days, the injections having been made for 10, 18, or 27 days respectively.

There were three main series of experiments: (1) injection of a saline extract of hen muscle (made from pectoral muscle); (2) injection of a saline extract of frog muscle; and (3) injection of a saline extract of hen liver.

There were also control experiments to indicate the course of muscle regeneration in the absence of injection. The results of the experiments are summarized in Table 1. The outcome of the control experiments was very consistent. From the 12th to the 30th day the place of the lesion changed only slightly: it gradually filled with fibrin and structureless masses resulting from the decay of the muscle fibres. No regenerative changes were noticed during this period (Plate 1, figs. A, B). We observed no formation of noticeable groups of myoblasts. However, on the 20th and 30th days one could observe some destruction of fibres in the region of the lesion and their concentration towards the middle of the wound, which might be considered as the beginning of a regenerative process.

When frogs were injected with a hen muscle-extract the results differed sharply from those of the controls. The wounds were already closed by the 10th day and the lesion was filled with decaying muscle fibres. By the 12th day these changes were still more prominent and there were some bundles of muscle in the lesion. Later, however, they decayed and on the 15th–17th day some nuclei surrounded with a layer of protoplasm emerged from the decaying fibres (Plate 1, fig. E). By the 20th day fully differentiated muscle-fibres and many myoblasts orientated along the future fibres were found in the region of the lesion (Plate 1, figs. C, D, F). A large number of myoblasts was also seen in the region of the lesion on the 30th post-operative day.

TABLE 1

Results of experiments on the stimulation of regeneration in frog muscle. Saline extracts unless otherwise stated

			Results	
Type of experiment	*Post-operative time*	*Number of specimens*	*Regeneration*	*No Regeneration*
	(days)			
Control	12	48	..	48
,,	20–22	11	..	11
,,	30	5	..	5
Hen muscle-extract	12	50	40	10
,, ,,	20–22	15	14*	1
,, ,,	30	10	10*	..
Frog muscle-extract	12	22	7	15
Hen liver-extract	12	7	1	6
Aqueous extract of hen muscle	12	18	15	3

* Clearly marked formation of new muscle-fibres was noticed.

Injections of a frog muscle-extract usually did not stimulate regeneration of frog muscles. Closing of the wound edges was noticed in only a few cases. The hen liver-extract showed no significant activity in the stimulation of regeneration.

The fact that the formation of myoblasts is preceded by the phase of fibre destruction, by some concentration of the muscle-fibres inwards towards the lesion, and by filling of the wound, makes us conclude that this phase is a necessary initial stage in muscle regeneration. Such morphological changes in the first phase of muscle regeneration are also described by other authors (Studitski & Striganova, 1951; Rumyantseva, 1956; Samsonenko, 1956). This supposition was also substantiated by the fact that in some of the control samples, especially at later stages, one could notice similar phenomena, though the process proceeded much less actively than in the stimulated wounds. This indicates that there was some acceleration of the regenerative processes under the influences of the extract.

STIMULATION OF LIVER REGENERATION BY SALINE EXTRACTS

These experiments were made mainly with frogs. Frog liver seemed suitable for the experiments as it is known that it regenerates incompletely in winter so that even on the 50th day there is only a connective-tissue scar (Zhenevskaya, 1954). Using spring frogs some investigators got a complete regeneration of the liver in rather a short time (Grigoriev, 1951). Our first experiments were made with winter frogs. Later, however, it was found that within the period of time taken by our experiments there was no special difference between winter and spring frogs, so that it was possible to disregard season (excluding the period of spawning).

The lesion was a hole with a diameter of 0·5 cm. made with a red-hot cylinder. The lesion in the control samples was filled with decaying cell elements and detritus on the 10th–12th day (Plate 2, figs. G, H). The picture in the control samples was very constant, and this gave an opportunity to establish the presence of a stimulating effect in the experiments rather exactly.

Experiments were made on the influence (1) of injected rabbit or hen liver saline extract, (2) of injected frog liver saline extract, (3) of injected hen muscle saline extract. The results are summarized in Table 2.

TABLE 2

Results of the experiments on stimulation of liver regeneration

Type of experiment	*Post-operative time* (days)	*Number of specimens*	*Results*		
			++	+	−
Control	12	37	..	..	37
Control	9	6	..	..	6
Extract of rabbit liver	12	8	6	1	1
Extract of hen liver	12	24	15	5	4
Extract of hen liver	9	4	..	4	..
Extract of frog liver	12	15	..	8	7
Extract of hen muscle	12	7	..	..	7
Aqueous extract of hen liver	9	10	8	2	..
Extract of hen liver heated to 60° C. for 5 minutes	12	5	..	..	5

++ Lesion completely filled with liver tissue, or area less than 5–6 mm.^{2} unfilled.
+ Area of the lesion not filled with liver tissue is less than 15 mm.^{2}
− The area of the lesion not filled with liver tissue is approximately 30–35 mm.^{2}

In a number of cases we measured the area which was not filled with liver tissue. The measurements were made in histological sections taken at different levels from the surface of the liver and an average magnitude characterizing the degree of filling of the lesion was thus obtained. There was a great difference only between the animals injected with rabbit or hen liver-extract and the controls. In the injected animals one could see amitotic cell-division already on the fifth day, as well as destructive phenomena and some ingrowth of the liver tissue at the edges of the wound. On the ninth day there was much amitotic cell-division and some mitotic cell-division (Table 3), (Plate 2, fig. K), the liver tissue had grown a great deal and only the centre of the wound was filled with structureless masses. By the 12th day in a number of cases the whole lesion was filled with normal liver tissue (Plate 2, figs. I, J) amongst which there were some compact groups of small, apparently recently formed liver cells.

TABLE 3

Effect of liver extracts on regenerating liver

Type of experiment	*Number of specimens*	*Number of amitotic divisions per 1,000 cells*	*Number of mitotic divisions per 1,000 cells*
Control	5	17·3	7
Extract of hen liver (saline or aqueous) . .	5	55·8	21
Extract of frog liver	2	25·0	10

Calculation of the number of amitotic and mitotic cell-divisions showed that there are appreciably more after injection of hen liver-extract than in the controls (Table 3). The influence of the frog liver-extract was much weaker. We found some slight growth of the liver tissue at the wound edges in some cases, but in most there was no clear difference from the controls. The injection of hen muscle-extract had no influence on the course of the frog liver regeneration.

TABLE 4

Results of experiments on liver regeneration of rabbits and guinea-pigs

Type of experiment	*Number of specimens*		*Results*			
			Stimulation		*No stimulation*	
	Rabbits	*Guinea-pigs*	*Rabbits*	*Guinea-pigs*	*Rabbits*	*Guinea-pigs*
Control	6	2	. .	. .	6	2
Liver extract . . .	13	2	10	2	3	. .
Liver extract heated to 60° C. plus ascorbic acid .	3	2	. .	. .	3	2

Similar results were obtained in experiments performed with rabbits and guinea-pigs (Table 4). A hole with a diameter of 12 mm. was made in the liver of these animals with a red-hot hollow steel cylinder with a cutting rim. The operated animals were injected subcutaneously with liver-extract of the same species. The injections of 1·5 ml. each were usually made three times a day for 9 days. The animals were dissected 10 days post-operatively. Altogether 19 rabbits and 6 guinea-pigs were operated upon, and of these 6 rabbits and 2 guinea-pigs were used as controls. The control animals had lesions filled with a friable mass of necrotic cells or, at best, with a connective-tissue scar. In 12 out of 15 cases the lesions of the experimental animals were almost completely filled with liver tissue.

STIMULATING ACTIVITY OF AQUEOUS EXTRACTS

In a special series of experiments we tried to stimulate the regeneration of frog liver and muscles with aqueous extract of the corresponding hen tissue. The lesions in liver and muscles were made and the extract was prepared as before. Sodium chloride, or the component salts of Ringer's solution, were added to the filtered extract so as to bring the concentration to 0·65 per cent. of NaCl or to that of Ringer's solution respectively. The extract prepared in this way contains only water-soluble substances.

The experiments showed that aqueous extracts had the same stimulating influence on the regenerative process of frog liver and muscle as the saline extracts (see Tables 1, 2).

INFLUENCE OF HEAT TREATMENT ON THE STIMULATING ACTIVITY OF THE EXTRACTS

It was shown in experiments with chick embryos that heating to 60° C. destroyed the stimulating influence of an extract on the homologous organ of an embryo (Tumanishvili, Jandieri, & Svanidze, 1956*a*). Similar results were obtained with the stimulating influence of extracts on liver regeneration. Hen liver-extract heated to 60° C. for 5 minutes did not stimulate regeneration of frog liver (Table 3). After heating to 60° C., liver extract did not stimulate regeneration of rabbit or guinea-pig liver in spite of the addition of ascorbic acid (a daily dose of 45 mg.) to the extract (Table 2). (It is known that ascorbic acid may accumulate in the liver of rodents. It is also known (Volinski, 1950) that ascorbic acid exerts some stimulating influence on liver regeneration.) Heating an extract to 60° C. always causes coagulation of some of its proteins.

DISCUSSION

Our experiments have shown that it is possible to stimulate regeneration of a tissue by an extract of the corresponding tissue. A similar phenomenon was obtained by Korkia (1956) with earthworms. She obtained stimulation of regeneration of the cutaneous-muscular body wall under the action of cutaneous-muscular extract, and she also obtained hyperplastic growth of the circular muscle-layer. The work of this author shows that the influence of tissue extracts on homologous tissue structures cannot be attributed only to mechanical displacement of the tissue components. It is obvious that the effect of the extracts is the stimulation of the regeneration process as a whole, at the basis of which, apparently, lies the increase of specific intracellular syntheses. This hypothesis is also confirmed by the facts which we have obtained. The increase of cell-division in the liver and the stimulation of the formation of myoblasts in muscles, which take place under the influence of extracts, can hardly be explained in any other way.

The action of extracts on the process of regeneration certainly has an elective tissue-specific character. Unfortunately it is impossible at present to say anything definite about the degree of specificity. However, the negative results of the attempts to stimulate regeneration of liver with a hen muscle-extract, and regeneration of muscles with a liver extract, demonstrate the elective action. The same experiments give reason to exclude the hypothesis that the injected protein extract acts as a nutrient. The nitrogen content of the extracts (determined by the Kjeldahl method) showed that all contained about the same quantity of protein: 35–40 mg. per 1 ml. of extract.

Tissue extracts are apparently characterized by specificity of two kinds: (1) the substances of the extract stimulate the reproduction only of certain cells and accelerate differentiation only in one direction (specificity of action), and (2) the stimulating agent interacts only with the homologous molecular substratum, resulting in its concentration in the homologous tissue (specificity of contact). Indeed, if the stimulating substances should be distributed in all tissues at random they would not so easily achieve an effective concentration in the place of the lesion. Such elective concentration of proteins in chick embryos was demonstrated by Ebert (1954, 1955) and Walter, Allman, & Mahler (1956).

These authors (Ebert made transplantations of organs to the chorio-allantois, and Walter *et al.* injected tissue homogenate into embryos) used methionine labelled with S^{35}. It was established that proteins containing radioactive methionine moved on the whole into the homologous organ. There is every reason to suppose that an analogous phenomenon takes place in the stimulation of regeneration of any tissue of adult animals.

As already mentioned, some authors observed an inhibiting action of substances isolated from tissues (Steuart—see Ebert, 1958*b*; Marsillii & Ciuti, 1953; Rose, 1955, 1958; Saetren, 1956; Weiss, 1952). We have never observed such an effect. Within the range of doses used in our experiments the effect always depended directly on the amount of the injected extract. But more recently it has been possible to show (in experiments with chick embryos) that the occurrence of a stimulating effect is closely connected with the quantity of extract injected. Stimulation of growth of the homologous organ takes place only in an optimum dose-range, and with further increase of the quantity injected the stimulating effect disappears completely. But even the largest dose does not depress the growth of the homologous organ (Tumanishvili, 1959*b*). These data contradict the hypothesis of 'specific inhibition' (Rose, 1955, 1958) and also make one rather careful about Weiss's hypothesis of the auto-regulation of tissue growth (1947, 1950). This hypothesis was based on data obtained in experiments with embryos, but it could be applied to regeneration of tissues of adult animals. It is likely that the small mobile extracellular elements to which Weiss ascribed inhibiting action would pass into an extract. When the extract is injected one should therefore first notice inhibition of regeneration; but this was not observed. It is logical, however, to suppose that there is an inhibiting as well as a stimulating agent. Which predominates in the effect of an extract apparently depends on conditions at present unknown.

The stimulating substances contained in extracts do not seem to be class specific. Indeed, regeneration of frog liver and frog muscle is well stimulated with extracts of the corresponding tissues of rabbit and hen. Furthermore, the use of extracts of homologous frog tissues gives non-significant results. This surprising result might be considered doubtful were it not repeatable with such consistency. There is no doubt that it demands further study. At present one may assume that the stimulating activity of an extract mainly depends on the properties of the tissue donor and not on the degree of species proximity, and that extracts of tissues of higher vertebrates show a bigger stimulating activity than extracts of frog tissues.

The high specificity of action and the results of heat treatment of the extracts suggest that the stimulating influence is mediated by intracellular proteins. Saetren (1956) made such an assumption about the substances liberated from macerated organs which influence specifically the mitotic activity of the homologous organ. The assumption is confirmed by the experiments with radioactive methionine made by Ebert (1954) and Walter *et al.* (1956). It is possible, however, that a substance forming a complex with proteins and precipitating together with the protein on heating has the stimulating ability. Possibly RNA is such an agent. Ebert's data (1958*a*) are very interesting in this respect. He showed in experiments with chick embryos that the microsomal fraction of the homologous organ is the most active in selective accumulation. The same fraction gives rise to specific inductions (Yamada, 1958; Hayashi, 1958; Niu, 1958) though it is not determined whether the active agent here is the protein component or the RNA of a ribonucleoprotein complex. The participation of DNA or of desoxyribonucleoproteins is less probable. It is known that DNA shows a very low resistance to ionizing radiations (Tongur, Golubeva, Diskina, Spitkovski, & Filippova, 1957; Budilova & Kuzin, 1957), while the stimulating agent is rather radio-resistant (Tumanishvili, 1959*a*). It is clear that the stimulating factor of a tissue extract is connected with a water-soluble and thermo-labile protein fraction.

There are some reasons for assuming that the substances described form one of the means of physiological regulation of tissue formation during the process of regeneration. The liberation of these substances increases in the first, destructive, phase of regeneration; this causes the second phase of regeneration, the phase of reconstruction and differentiation, to begin and develop. Such an idea agrees with the fact that the second phase of regeneration proceeds the better the more intensive the first phase is (Polezhaev, 1947, 1956; Umanski & Kudokozev, 1951, 1952; Kudokozev, 1957; Rose, 1944).

SUMMARY

1. By means of systematic injections of tissue extracts one may obtain stimulation of regeneration of muscles (in frogs) and liver (in frogs, rabbits, guinea-pigs). Such extracts, however, apparently only influence homologous tissue.

2. The influence of extracts does not show any class specificity. On the contrary, the stimulation of regeneration of frog muscle and liver is more effectively produced by corresponding extracts of hen tissues than by those of the frog.

3. Aqueous extracts are as active as those obtained with isotonic saline.

4. Heating to 60° C. for 5 minutes inactivates the extract.

5. The analysis of our data and of those published elsewhere leads to the conclusion that the stimulating agent is a water-soluble protein fraction, or that it is some substance (e.g. RNA) forming a rather strong complex with this fraction.

RÉSUMÉ

Action stimulatrice des extraits de tissu sur les processus de régénération

1. Des injections systématiques d'extraits de tissus ont pour effet de stimuler la régénération des muscles (chez les grenouilles) et du foie (chez les grenouilles, les lapins, les cobayes). Ces extraits ne paraissent cependant avoir d'action que sur des tissus homologues.

2. L'action des extraits ne montre aucune spécificité de classe. Au contraire, la stimulation de la régénération du muscle et du foie de grenouille est plus efficace quand elle est provoquée par les extraits correspondants des tissus de poule que par les extraits de grenouille.

3. Les extraits aqueux sont aussi actifs que les extraits préparés avec une solution saline isotonique.

4. Le chauffage à 60° C. pendant 5 minutes inactive l'extrait.

5. L'analyse de ces faits et des données publiées ailleurs permet de conclure que l'agent stimulateur est une fraction protéique soluble dans l'eau, ou une substance (par exemple le RNA) formant avec cette fraction un complexe assez stable.

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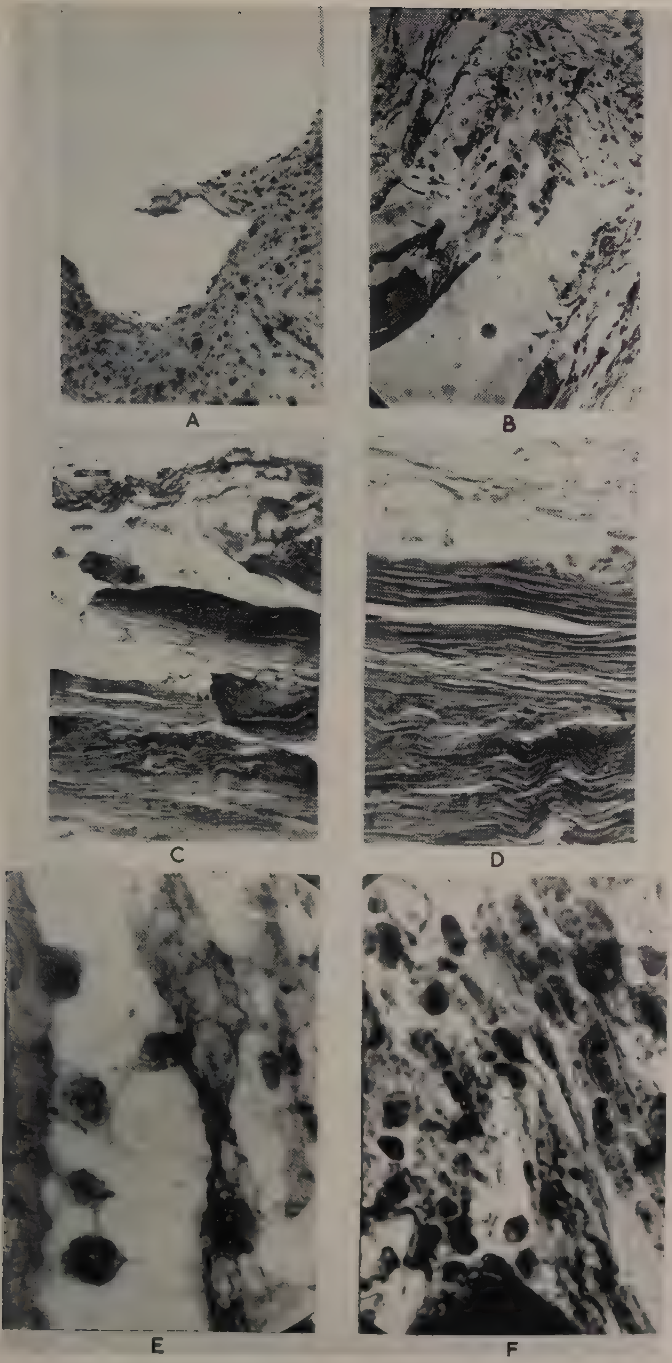
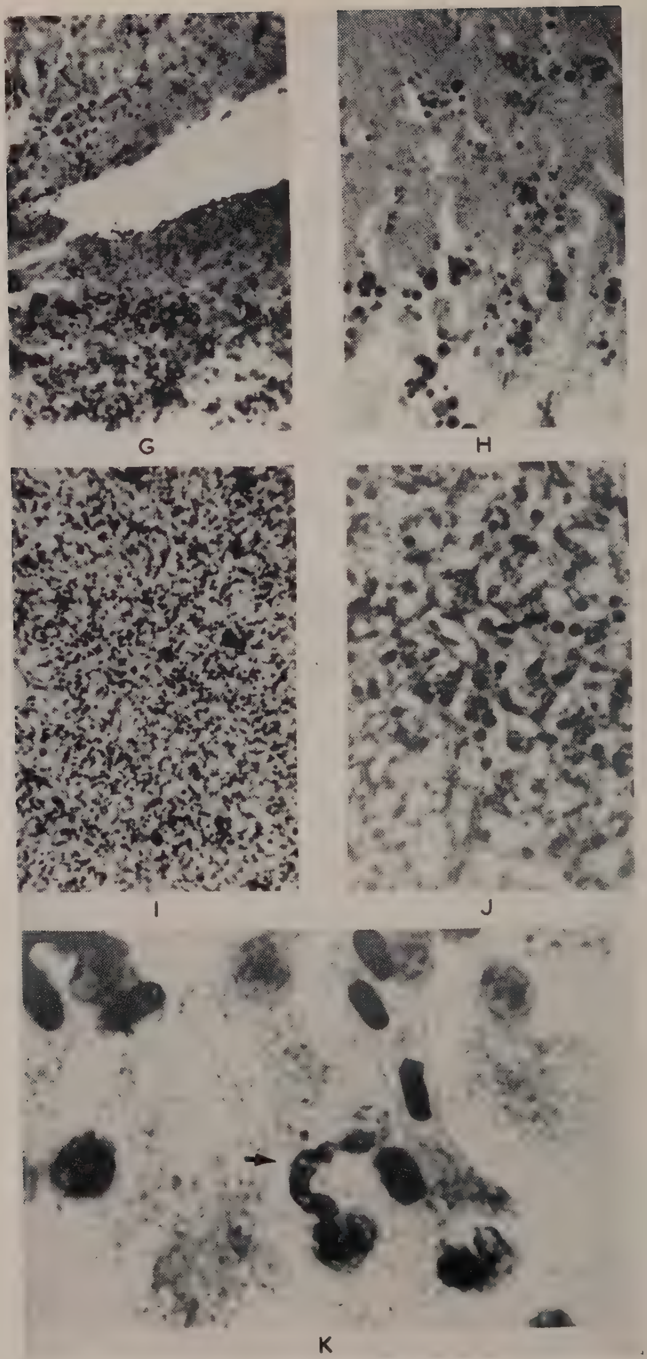


G. D. TUMANISHVILI

Plate 1



G. D. TUMANISHVILI

Plate 2

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EXPLANATION OF PLATES

Plate 1

Fig. A. Frog muscle. The region of the lesion on the 20th post-operative day in a non-injected control. A 'funnel' which appeared as a result of the lesion is seen. The main part of it is unfilled; the rest is filled with fibrin and structureless material. Heidenhain's haematoxylin; oc. ×15, obj. ×6.

Fig. B. The same preparation as shown in fig. A, at the bottom of the 'funnel'. The ends of the cut fibres are seen. They are almost unchanged. Heidenhain's haematoxylin; oc. ×10, obj. ×40.

Fig. C. Frog muscle. The region of the lesion on the 20th post-operative day. Hen muscle-extract had been injected. The area formerly occupied by the 'funnel' is shown. Some muscle-fibres sprouting into the region of the lesion may be seen. Heidenhain's haematoxylin; oc. ×15, obj. ×6.

Fig. D. Detail from fig. C at higher magnification. Transversely striped newly formed fibres are seen. Heidenhain's haematoxylin; oc. ×10, obj. ×40.

Fig. E. Frog muscle. The region of the lesion on the 17th post-operative day. Hen muscle-extract had been injected. One can see a decaying fibre with nuclei coming out of it. Mallory; oc. ×15, obj. ×90.

FIG. F. Frog muscle. The region of the lesion on the 20th post-operative day. Hen muscle-extract had been injected. One can see longitudinally orientated myoblasts. Mallory; oc. × 15, obj. × 60.

PLATE 2

FIG. G. Frog liver. The region of the lesion on the 12th post-operative day in a control specimen. The edges of the hole which formed as a result of the lesion are seen, so is part of a structureless mass filling it. The cells of the edge have also suffered destruction. Heidenhain's haematoxylin; oc. × 15, obj. × 6.

FIG. H. The same preparation as shown in fig. G, showing the structureless mass filling the hole at a higher magnification. Heidenhain's haematoxylin; oc. × 15, obj. × 40.

FIG. I. Frog liver. The place of the lesion on the 12th post-operative day. Hen liver-extract had been injected. Heidenhain's haematoxylin; oc. × 15, obj. × 6.

FIG. J. Detail from fig. I at a higher magnification; oc. × 15, obj. × 20.

FIG. K. Frog liver. The place of the lesion on the 9th post-operative day. Hen liver-extract had been injected. An amitotically dividing nucleus is seen (indicated by an arrow). Heidenhain's haematoxylin; oc. × 15, obj. × 60.

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